University of Alberta

Simultaneous Quantification of Chiral and Achiral Pharmaceuticals in Surface Waters and Wastewaters Using Chiral Liquid Chromatography- Tandem Mass Spectrometry

by

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Abstract

Chiral liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) was used to measure pharmaceuticals in surface and wastewaters. Analytes measured were chiral non-steroidal anti-inflammatory drugs (NSAIDs) ibuprofen, naproxen, fenoprofen, ketoprofen; β-blockers metoprolol, atenolol and propranolol; and selective serotonin re-uptake inhibitor fluoxetine. Achiral drugs measured were NSAIDs diclofenac and indomethacin; lipid regulators clofibric acid and gemfibrozil; and stimulant caffeine. Water samples were concentrated by solid phase extraction and target analytes separated using Chiralpak AD-RH and Chirobiotic V columns. Recoveries (60 to 108%), linearity (0.9739 to 0.9969) and limits of detection (LOD) (0.01 to 39 ng/L) were compared to previous measurements in environmental waters. Stereoisomers of chiral targets were separated (resolution factors between 0.6 and 0.9) and enantiomer fractions (EFs) were determined. Analytes were detected in most environmental samples. Non-racemic EFs were seen in WWTP effluent samples and in Lac la Biche WWTP influents. No chiral targets were seen in the surface water samples.

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Chapter 1: Introduction

The importance of surface waters in everyday life is leading to a growing concern over the widespread occurrence of chiral and achiral pharmaceuticals in the aquatic environment (Huber et al. 2003). Although some early studies illustrated the presence of certain pharmaceuticals in the environment, the issue has not been largely studied until recently (Buser et al. 1998b; Daughton et al. 1999; Halling-Sorensen et al. 1998). Given that the amount of prescription and non-prescription pharmaceutical drugs used and produced each year exceeds hundreds of metric tons, the occurrence and fate of these drugs in the environment is an important and emerging concern (Metcalfe et al. 2003a). Such a large volume of use coupled with the potential for release into the environment is becoming an environmental issue that may require legislative intervention (Metcalfe et al. 2003b). Fortunately, the first small steps are being taken and the U.S. Food and Drug Administration is now requiring environmental risk assessments for new drugs that have predicted introduction concentrations estimated to be greater than 1µg/L (Metcalfe et al. 2003b). Due to the potentially different pharmacological, pharmacokinetic and toxicological effects of individual pharmaceutical isomers that are distinguishable by biological systems, the chiral nature of pharmaceuticals cannot be neglected and separation of the optical isomers is essential (Pehourcq et al. 2001). This separation requires either enantioselective synthesis or analytical methods that are able to qualitatively and quantitatively separate each enantiomer from other compounds present in a given sample (D'Orazio et al. 2005; Hamon et al. 1995).

Pharmaceuticals enter the environment through human excretion of metabolized and unmetabolized drugs, veterinary usage, improper disposal, and wastewater treatment plant (WWTP) effluents (Richards et al. 2004). They enter the soil and subsurface in a number of ways, including filtration through river banks, artificial groundwater discharge, naturally occurring influent groundwater flow conditions and leaky sewage systems (Shen et al. 2005). Given a large enough production and suitable physicochemical properties, pharmaceuticals may reach detectable concentrations (ng/L), although a number of factors affect the likelihood of a drug reaching the environment (Buser et al. 1998c; Cahill et al. 2004). These include the quantity sold, the pharmacokinetic behaviour in humans, the rates of chemical, microbial and phototransformations within the environment and degradation occurring in WWTP (Metcalfe et al. 2003a). A compound with high aquatic mobility may easily make its way into rivers, streams and groundwater and some compounds may be capable of long-range transport (Ashton et al. 2004; Buser et al. 1998c).

Despite many natural modes of degradation, a constant input of pharmaceuticals into the environment can lead to a high steady-state concentration similar to that found for persistent compounds, such as DDT (Lam et al. 2004). The continual discharge of generally persistent xenobiotics into the environment, be it DDT, pharmaceuticals or other environmental pollutants, can result in an accumulation that increases their concentration over time despite easy degradation (Drillia et al. 2005; Lam et al. 2004). Since pharmaceuticals are designed to elicit responses in both humans and animals at low concentrations and often at a very specific target, it is important to know their environmental fate and whether they are accumulating or being removed (Comoretto et al. 2005). Although the concentrations of pharmaceuticals found in the environment may be too low to cause any acute toxic effects, these low concentrations may have subtle long-term effects on wildlife and humans (Ashton et al. 2004; Miao et al. 2002). The impact such pharmaceuticals may have on the environment and the organisms in the aquatic ecosystem is still largely unknown, despite the large volume of compounds released every day (Richards et al. 2004). Given the wide range of pharmaceuticals and their numerous modes of action, many concerns remain about organisms inhabiting these waters (Richards et al. 2004). The unknown consequences resulting from the presence of such compounds suggests that environmental waters, as well as drinking water, should be free of xenobiotic compounds to minimize the risk of unpredictable long-term effects (Huber et al. 2003).

The objective of this thesis is to develop analytical methodology to measure a selected group of chiral and achiral human-use pharmaceuticals likely to be present in the Alberta aquatic environment detectable and environmentally relevant concentrations (ng/L). The following describes how the research in this thesis is organized and how each part relates to the overall hypothesis of chiral pharmaceutical occurrence and fate in the Alberta environment.

Chapter 2 is a literature review that encompasses four parts. Chapter 2.1 discusses how pharmaceuticals are being released into the environment and where they are found. It focuses on a group of thirteen pharmaceuticals belonging to the non-steroidal anti-inflammatory drug (NSAID), lipid regulating, stimulant and selective serotonin re-uptake inhibitor (SSRI) and beta-blocking classifications. The pharmaceuticals were chosen based on their presence in the literature and most have been previously investigated in various studies throughout the world, including Eastern Canada (Metcalfe et al. 2003a; Metcalfe et al. 2003b). To date, only one peer reviewed study in the primary literature of which we are aware has investigated pharmaceuticals in Alberta, specifically in Calgary (Metcalfe et al. 2003a).

Chapter 2.2 discusses the pharmacology and use of each target pharmaceutical investigated. An understanding of the pharmacology and physical properties of a drug is important for the prediction of environmental occurrence. The structures are given along with a table of properties. The use, pharmacology, metabolites and toxicokinetics are discussed and arranged by drug and class.

Chapter 2.3 discusses the environmental occurrence and fate processes of the target pharmaceuticals. The mass transfer processes, including sorption and partitioning, and the transformation processes, including photodegradation, thermal degradation and biotic and abiotic degradation, that pharmaceuticals may undergo in the environment are discussed. The contribution due to WWTPs is looked at and specific environmental findings about which of the target drugs are being found, and where, are given.

Chapter 2.4 discusses the analytical procedures that have been used to measure pharmaceuticals in the environment. Extraction from environmental matrices and chiral and achiral separation are discussed at length. Detection techniques and the difficulties associated with them are discussed along with potential solutions. Analytical data for the target pharmaceuticals is presented and interpreted.

Chapter 3 presents the analytical techniques used in the detection of the specific target pharmaceuticals. Methods development and validation is discussed. Specific chiral HPLC columns were used with LC-MS/MS and the results are discussed, and a preliminary survey of the environmental occurrence of chiral and achiral pharmaceuticals in the Alberta environment is presented. Environmental water samples were taken from the Lac la Biche WWTP and the Gold Bar WWTP at Edmonton and analyzed using the validated methods described in Chapter 3. These results are discussed and the future direction for continuance of this research is presented.

Chapter 2: Literature Review

2.1.1 Release of Pharmaceuticals in the Environment

Natural waters are used for ecosystems, drinking water and food resources, irrigation and recreational activities. As such, there is a growing concern about the occurrence of pharmaceuticals in the aquatic environment (Buerge et al. 2003; Huber et al. 2003). Given a large enough production and suitable physicochemical properties, pharmaceuticals may reach detectable concentrations (ng/L) within the environment and may be detectable in rivers and lakes if the drugs are mobile in the aquatic environment (Buser et al. 1998c; Cahill et al. 2004). The concern is that detectable concentrations of pharmaceuticals may cause negative or toxic effects in aquatic organisms, humans and other biota, including plants, algae and bacteria.

A number of factors affect the likelihood of a drug reaching the environment, including the quantity sold, the pharmacokinetic behaviour in humans as well as the rates of chemical, microbial and phototransformations within the environment and degradation occurring in a WWTP (Metcalfe et al. 2003a). Pharmaceuticals are known to enter the environment through human excretion of metabolized and unmetabolized drugs, veterinary usage, improper disposal, and WWTP effluents. Pharmacies will accept unused or expired medications for proper disposal but undoubtedly many medications end up in the garbage or the sewage system. In spite of this, the improper disposal of unwanted or expired drugs is believed to be a minor source of pharmaceutical contamination in the environment (Bendz et al. 2005).

Human and veterinary excretion and subsequent transport through sewage and WWTP are considered to be the main sources of pharmaceuticals entering the environment (Bendz et al. 2005). Large volumes of untreated sewage enter US and Canadian surface waters daily. This untreated sewage and WWTP effluents are known to be an important source of pharmaceuticals and non-prescription drugs in rivers and streams (Metcalfe et al. 2003b; Richards et al. 2004). Despite degradation in the environment by biotic or abiotic processes, the continual infusion of pharmaceuticals into the aqueous environment via WWTP effluents may result in high steady-state concentrations that resemble the persistence seen for persistent organic pollutants (Ferrari et al. 2004). Non-quantitative removal from WWTP during the water treatment process has been identified as a major source of environmental discharge (Brooks et al. 2005; Doll et al. 2004; Ferrari et al. 2004). Some WWTPs in large Canadian cities, including Montreal (Quebec), Toronto (Ontario), Winnipeg (Manitoba), Calgary (Alberta) and Vancouver (British Columbia), have shown poor removal rates of drugs (Metcalfe et al. 2003a). Cold weather during the winter months has also been shown to decrease sewage treatment efficiency (Metcalfe et al. 2003a). Additionally, seasonal differences may be seen due to the use of pharmaceuticals to treat the symptoms of viral infections (e.g. colds, influenza) (Metcalfe et al. 2003a).

A compound with high aquatic mobility may be able to make its way into rivers, streams and groundwater, and may thus be capable of long-range transport (Ashton et al. 2004; Buser et al. 1998c). Water contamination is dependant on a number of factors, including the type of soil through which the water filters and the physico-chemical properties of the pharmaceuticals, such as water solubility, vapour pressure and partition coefficients. Animal waste from pastures or confined animal-feeding operations is also considered a possible source (Cahill et al. 2004). Surface waters can be contaminated by the direct discharge of livestock feces to the ground, while ground water can be contaminated by the leakage of waste in lagoons (Cahill et al. 2004). Due to partitioning to sediments, environmental stability and the sheer number of possible origins, identification of the specific point sources in which pharmaceuticals enter the environment can be difficult (Gardinali et al. 2002). Once in the environment, pharmaceuticals are able to enter the soil and subsurface in a number of ways, including filtration through river banks, artificial groundwater discharge, naturally occurring influent groundwater flow conditions and leaky sewage systems (Shen et al. 2005).

Because the aquatic environment acts as a sink for many persistent pollutants, the presence of pharmaceuticals raises questions about the impact such residues will have on the environment (Andreozzi et al. 2003; Buser et al. 1998b). Lakes and seas receive inputs of anthropogenic compounds from agricultural, industrial and other human activities via rivers and the atmosphere (Buser et al. 1998b). It has been argued that pharmaceutical concentrations in receiving waters are too low to have an impact on the organisms living there (Drillia et al. 2005). This argument needs to be reconsidered because the continuous discharge of xenobiotics can result in an environmental persistence in spite of easy degradation (Drillia et al. 2005; Lam et al. 2004). It is still unclear what consequences will result from low doses of pharmaceuticals that are designed to elicit a response in humans and animals at low concentrations (Comoretto et al. 2005). The low environmental concentrations typically found for pharmaceuticals may not lead to acute toxicity but may be problematic if non-target organisms have receptors sensitive to certain residues or combinations of drugs that have a common mode of action (Lin et al. 2005). There is concern that mixtures of pharmaceuticals may lead to synergistic effects in which the combination increased the potency of one or all drugs present (Lin et al. 2005; Richards et al. 2004). Data on exposure and the effects pharmaceuticals have on non-target aquatic living organisms are still needed and this lack of ecotoxicological data is a hurdle that must be overcome in order to asses the environmental hazard of therapeutic pharmaceuticals (Andreozzi et al. 2003; Isidori et al. 2005). It is believed that fish responses to pharmaceuticals may be predicted based on mammalian pharmacological safety information if the fish species possesses a receptor targeted by a specific pharmaceutical (Brooks et al. 2005).

2.1.2 Chirality

Many current use pharmaceuticals are chiral in nature. A chiral molecule consists of two non-super-imposable mirror images known as enantiomers that have identical physical and chemical properties except for the direction in which they rotate plane polarized light. The big difference, as it concerns pharmaceuticals, is the profoundly different pharmacological properties that may be exhibited by the enantiomers (Lees et al. 2003; Shen et al. 2002). The body is a chiral environment and as such, it is able to differentiate between the enantiomers. The pharmacokinetic characteristics of each enantiomer, including differences in the absorption, distribution, metabolism and excretion must be considered when a new chiral drug is developed and used (Chen et al. 2005; Shen et al. 2002). In the past, most chiral drugs were marketed as racemates due to the difficulty of performing enantiomer separations and synthesizing individual enantiomers. This was the case for thalidomide in the 1950s, where one enantiomer exhibited severe teratogenic effects including limb and internal organ malformations before being withdrawn from clinical practise (Meyring et al. 2000). However, some chiral drugs are still administered clinically as racemates today (Chen et al. 2005; Hamon et al. 1995). The pharmaceutical industry has been addressing this issue by producing single enantiomer drugs when it is known the enantiomers have different pharmacological and biological activities (Chen et al. 2005; D'Orazio et al. 2005). There has been increasing attention paid to the asymmetric synthesis of non-racemic chiral drugs as well as increasing interest in the stereospecific separation of enantiomers since the advent of chromatographic enantioseparation techniques and the availability of chiral stationary phases (Berzas et al. 2004; Buser et al. 1999; Chen et al. 2005; D'Orazio et al. 2005; Hamon et al. 1995; Moraes de Oliveira et al. 2005a; Moraes de Oliveira et al. 2005b; Pehourcq et al. 2001; Shen et al. 2002). Chiral separation is discussed in Chapter 2.4.

2.1.3 Metabolites

Once administered, pharmaceuticals are degraded in the body to a variety of metabolites with varying efficiency. Identification of these metabolites can help determine any potentially toxic degradation products and is an important step in developing safe and effective drugs (Bandu et al. 2004). These metabolites are able to easily enter the environment through human excretion of metabolized and unmetabolized drugs and through veterinary usage (Richards et al. 2004). Once released into the environment, both the metabolites and unmetabolized parent drugs are free to undergo a variety of chemical, physical and biological processes, including microbial transformations and abiotic degradation such as hydrolysis and photolysis (Buser et al. 1998b; Isidori et al. 2005). Often the products of these reactions can be more harmful than the parent compound, and certain metabolites like clofibric acid (from clofibrate) are active in sediments (Buser et al. 1998b; Isidori et al. 2005). This toxicity can have significant adverse effects on biological species, highlighting the importance of ecological testing of pharmaceuticals (Isidori et al. 2005).

Chapter 2.2 Pharmacology and Use

2.2.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are a commonly used class of pharmaceuticals. They have analgesic (pain relief), anti-pyretic (fever reduction) and anti-inflammatory properties that make them useful in the symptomatic treatment of such diseases as rheumatoid arthritis and osteoarthritis

(Al-Saidan 2004; Aresta et al. 2005; Castro et al. 2000; Wang et al. 2004; Wang et al. 2005). Certain NSAIDs, like naproxen and indomethacin, have both human and veterinary applications (Metcalfe et al. 2003a; Novakova et al. 2005). In humans, NSAIDs inhibit the cyclooxegenases (COX), although the exact mode of action is not clearly understood (Cleuvers 2004; Wang et al. 2004). Inhibition of cyclooxegenases prevents the biosynthesis of prostaglandins from arachidonic acid, a reaction that is responsible for pain and inflammatory responses (Cleuvers 2004; Kenawi et al. 2005; Lilley et al. 2001). This inhibition of prostaglandin synthetase is responsible for the anti-inflammatory and analgesic effects of NSAIDs (Cleuvers 2004; Savaser et al. 2004). The *S*-(+) arylpropionates, such as ketoprofen, ibuprofen, fenoprofen and naproxen, are much more potent inhibitors of COX than are the *R*-(-) arylpropionates and have different pharmacokinetics (Castro et al. 2000; Hamon et al. 1995; Igarza et al. 2002; Lees et al. 2003; Lin et al. 2004; Montoya et al. 2004; San Martin et al. 2002; Sevoz et al. 1999). Inhibition of the prostaglandins that protect cells in the gastrointestinal tract can lead to internal bleeding and ulceration, one of the most important side effects of NSAIDs (Aresta et al. 2005; Cleuvers 2004; Wang et al. 2005). Other negative side effects associated with NSAIDs include gastropathy, tinnitus, nausea, degenerative inflammatory liver alterations and both functional and morphological renal changes (Aresta et al. 2005; Lilley et al. 2001; Schwaiger et al. 2004).

Six NSAIDs are included in the list of target compounds (Fig. 2.1, Table 2.1) investigated in this thesis and discussed in detail: ibuprofen, diclofenac, naproxen, indomethacin, fenoprofen and ketoprofen. Of these, ibuprofen, fenoprofen, ketoprofen and naproxen are chiral pharmaceuticals.

2.2.1.1 Ibuprofen is a white powder sold in both tablet and solution forms under many trade names, including Advil™ and Motrin™. Available in prescription and non-prescription formulations, ibuprofen is one of the most commonly used pharmaceuticals in the world. The over-the-counter availability of this drug makes estimations of its annual usage difficult, although Germany has reported estimates of 180 tons/year (Cleuvers 2004).

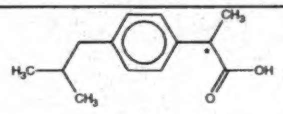
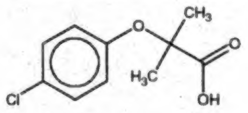
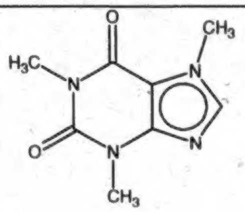
Ibuprofen has analgesic (pain relief), anti-pyretic (fever reduction) and anti-inflammatory (inflammation reduction) properties. Ibuprofen is excreted largely as the parent compound, at 70 to 80% of the given dose (Buser et al. 1999). Peak levels are generally found in the blood one to two hours after ingestion of the tablets, and the drug duration is 4-6 hours (Adams et al. 1967; Lilley et al. 2001). No differences are noticed in absorption whether the drug is taken with or without food (Adams et al. 1967). It has shown comparable results to those of indomethacin in the treatment of rheumatoid arthritis (Royer et al. 1975). The side effects and the adverse effect profile of ibuprofen are relatively safe, leading to its popularity as the most frequently used propionic acid NSAID (Lilley et al. 2001).

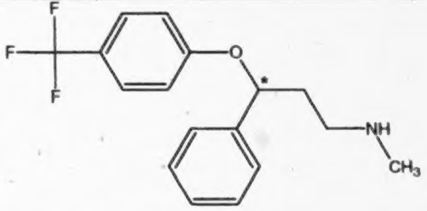
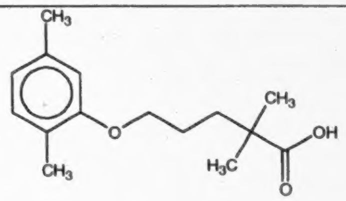
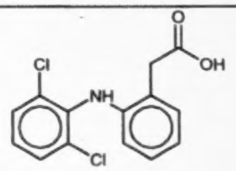
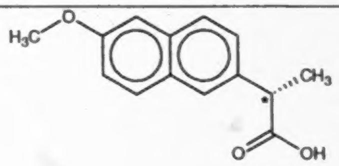
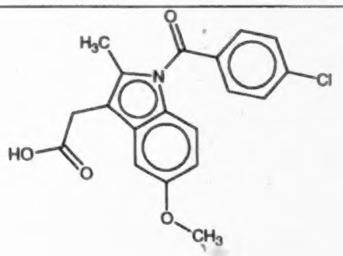
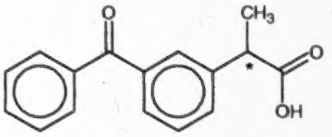
Ibuprofen is a chiral compound and is stereoselectively degraded by the body into two main metabolites, (+)-2-[*p*-(2-hydroxymethyl-propyl) phenyl]-propionic acid and (+)-2-[*p*-(2-carboxypropyl) phenyl] propionic acid (Fig. 2.2) (Glowka et al. 2005; Moraes de Oliveira et al. 2005a; Weigel et al. 2004a). The hydroxy-metabolite is the major metabolite found in sewage, while the carboxy-metabolite is the major metabolite found in seawater (Weigel et al. 2004a). Ibuprofen is excreted mainly in urine as the free parent (1%), conjugated parent (14%), hydroxy-metabolite (25%) and carboxy-metabolite (37%) (www.rxlist.com 2003). In humans, the excretion of *S*-(+) ibuprofen is higher than that of *R*-(-)-ibuprofen (Lees et al. 2003; Moraes de Oliveira et al. 2005b).

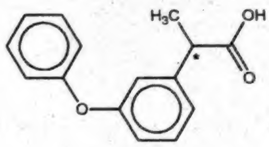
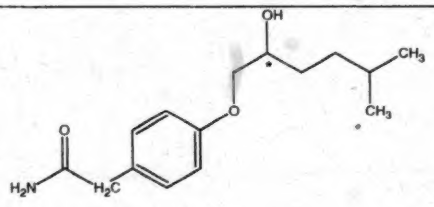
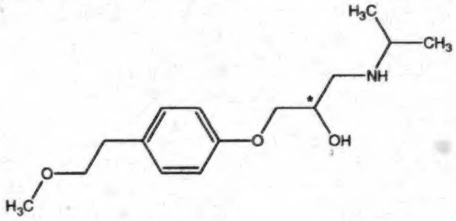
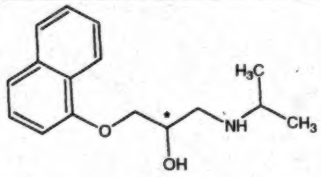
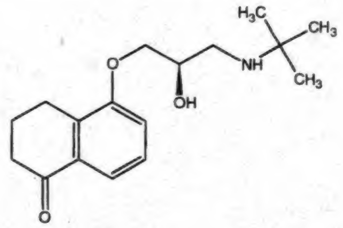
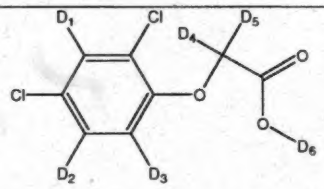
R-(-) ibuprofen is converted to S-(+) ibuprofen with 53-60% efficiency and results in higher concentrations of the S-(+) isomer in plasma and urine (Lin et al. 2004; Moraes de Oliveira et al. 2005a; Moraes de Oliveira et al. 2005b). The biotransformation of R-(-) ibuprofen to S-(+) ibuprofen results from an inversion of configuration at the chiral center (Fig. 2.3) (Moraes de Oliveira et al. 2005a).

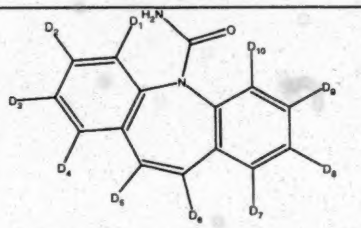
Fig. 2.1 Pharmaceutical Structures

*** marks the chiral center**

Structure	**Name (CAS)**	**Class**
	Ibuprofen (15687-27-1)	NSAID
	Clofibric Acid (882-09-7)	Lipid Regulator
	Caffeine (58-08-2)	Stimulant

Structure	Name (CAS)	Class
	Fluoxetine (54910-89-3)	SSRI
	Gemfibrozil (25812-30-0)	Lipid Regulator
	Diclofenac (15307-86-5)	NSAID
	Naproxen (22204-53-1)	NSAID
	Indomethacin (53-86-1)	NSAID
	Ketoprofen (22071-15-4)	NSAID

Structure	Name (CAS)	Class
	Fenoprofen (29679-58-1)	NSAID
	Atenolol (29122-68-7)	β-Blocker
	Metoprolol (65392-17-7)	β-Blocker
	Propranolol (318-98-9)	β-Blocker
	(+)-Levobunolol (47141-42-4)	β-Blocker Positive mode internal standard
	D_6-2,4-D (94-75-7)	Pesticide Negative mode internal standard

Structure	Name (CAS)	Class
	D_{10}- Carbamazepine (298-46-4)	Anti- convulsant Positive mode internal standard

2.2.1.2 Diclofenac is a yellowish-white crystalline drug sold by prescription in pill form under the trade names Cataflam™, Voltaren™ and Voltaren-XR™. Diclofenac is a highly prescribed drug in Europe, with 16,000 kg/year used in France and 75,000 kg/year used in Germany (Ferrari et al. 2004). It is one of the most commonly found drugs in WWTP and surface waters (Schwaiger et al. 2004). Diclofenac was the first phenylacetic acid derivative to be approved for drug use and has anti-inflammatory, anti-pyretic, analgesic and anti-rheumatic properties (Schwaiger et al. 2004; Wang et al. 2004). Within 30 minutes of dosing, diclofenac can be detected in the blood and reaches a maximum blood concentration of 0.7 to 1.5 mg/L in 1.5 to 2.5 hours (Savaser et al. 2004). However, this maximum is affected by the presence of food and the specific diclofenac formulation (Savaser et al. 2004). Diclofenac has an oral bioavailability of about 60% and an excretion half-life of 1.1 to 1.8 hours (Savaser et al. 2004; Skidmore-Roth 2003). During first-pass metabolism, drugs are absorbed directly from the small intestine and transported to the liver for metabolism (Skidmore-Roth 2003; Wang et al. 2004). Only 50% of diclofenac is available after first-pass metabolism in the liver, although it is well absorbed

(Skidmore-Roth 2003). When taken with food, the efficiency of absorption is not affected, but the rate of absorption is delayed by one to four hours with decreased gastric symptoms (Skidmore-Roth 2003; www.rxlist.com 2003).

The three brand name diclofenac formulations mentioned above vary according to their release characteristics and the positive ion associated with the diclofenac anion (www.rxlist.com 2003). Cataflam™ Immediate-Release tablets release diclofenac in the stomach, while Voltaren™ releases the drug in the duodenum (into which the stomach opens), because of higher ambient pH in the duodenum (www.rxlist.com 2003). Voltaren-XR™ releases diclofenac into the duodenum over an extended period (www.rxlist.com 2003).

Diclofenac metabolizes to form glucuronide and sulfate conjugates that are excreted 65% in the urine and 35% in bile secretions (www.rxlist.com 2003). One source reports diclofenac is excreted from humans 15% unchanged (Schwaiger et al. 2004). Another author found little or no parent compound is excreted unconjugated, although 5-15% of the dose is excreted as conjugates of unchanged diclofenac in urine and bile (www.rxlist.com 2003). A third study reported that <1% of the parent is excreted unchanged (Al-Saidan 2004). The main metabolite's conjugate accounts for 20-50% of the parent dose in both urine and bile (www.rxlist.com 2003). Degradation of diclofenac preparations can be affected by temperature, humidity and light (Galmier et al. 2005). Possible degradation processes include oxidation, hydrolysis, and dehydration and adduct formation (Galmier et al. 2005).

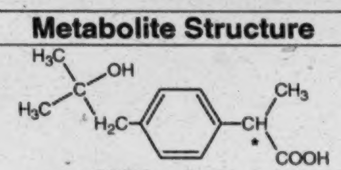
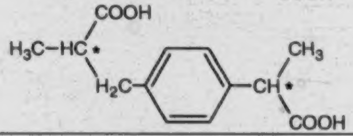
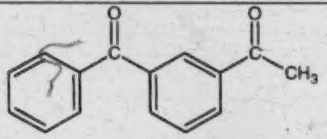
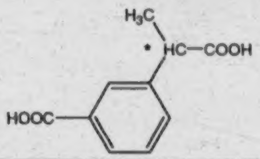
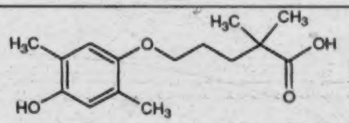
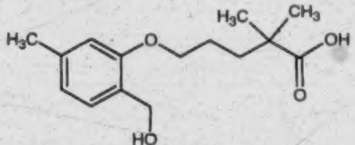
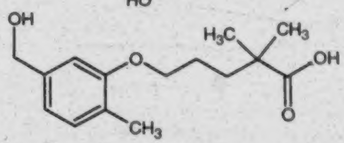
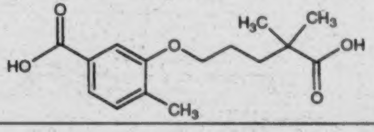
Table 2.1 Physical Properties

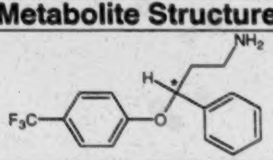
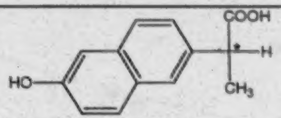
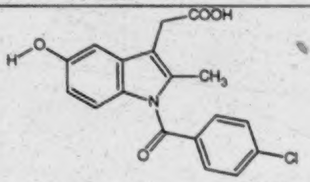
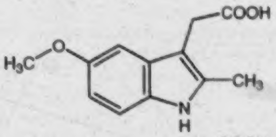
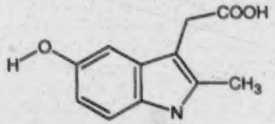
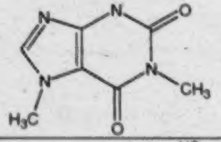
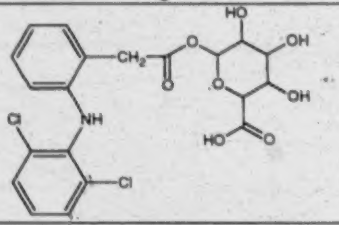
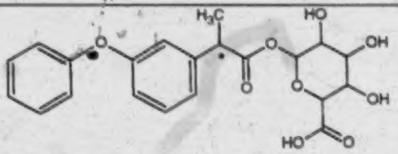
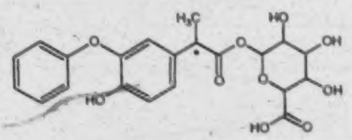
Drug	Molecular Formula	Molecular Weight (g/mol)	pKa	$\log K_{ow}$	Melting Point (°C)	Boiling Point (°C)	Henry's Law Constant (atmxm^{3}mol^{-1})	Flash Point (°C)	Enthaply of Vaporization (kJ/mol)	Solubility (mg/L)	Vapor Pressure (Pa)
Ibuprofen	$C_{13}H_{18}O_2$	206.27	4.52 [a]	3.5 [a]	74.87	319.6 ± 11.0	1.5×10^{-7} [d]	216.7 ± 25.9	59.25 ± 3.0	21 [a]	2.47×10^{-5} [a]
Clofibric Acid	$C_{10}H_{11}O_3Cl$	214.65	3.18 ± 0.20	2.724±0.272	123°C	324.1 ± 17.0	2.19×10^{-8} [d]	149.8 ± 37.7	59.76 ± 3.0	582.5 [i]	
Caffeine	$C_8H_{10}N_4O_2$	194.19	14 [g]	-0.081 ± 0.351	234.0-236.5	416.8 ± 37.0	1.9×10^{-9} [j]	205.9 ± 47.7	67.01 ± 3.0	13500 [b]	1.333×10^{-6} [j]
Fluoxetine	$C_{17}H_{18}F_3NO$	309.33	10.05 ± 0.20	4.352 ± 0.308	156-158	395.1 ± 32.0		192.8 ± 45.2	64.52 ± 3.0		
Gemfibrozil	$C_{15}H_{22}O_3$	250.33	4.75±0.20	4.387±0.487	58-61	394.7±30.0		141.6±32.5	68.00±3.0		
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	296.15	4.16 [a]	4.51 [a]	180	412.0±40.0	4.73×10^{-12} [d]	203.0±49.2	70.07±3.0	2.37 [a]	8.17×10^{-6} [a]
Naproxen	$C_{14}H_{14}O_3$	230.26	4.40±0.20	2.998±0.239	156-157	403.9±20.0	3.39×10^{-10} [d]	154.5±27.5	69.10±3.0		
Indomethacin	$C_{19}H_{16}ClNO_4$	357.79	4.17±0.20	3.105±0.372	163	574.6±50.0		301.3±54.2	90.56±3.0		
Ketoprofen	$C_{16}H_{14}O_3$	254.28	4.23±0.20	2.814±0.326	95-96	431.3±28.0	2.12×10^{-11} [d]	228.8±36.9	72.41±3.0		
Fenoprofen	$C_{15}H_{14}O_3$	242.27	4.20±0.20	3.844±0.326		381.3±25.0		141.7±30.0	66.41±3.0		
Atenolol	$C_{14}H_{22}N_2O_3$	266.34	9.6	0.16 [k]	146-148		1.37×10^{-18} [k]			26500	3.893×10^{-8}
Metoprolol	$C_{15}H_{25}NO_3$	267.37	9.7	1.88 [k]	96		1.4×10^{-13} [k]			$>1.00 \times 10^6$	3.84×10^{-5}
Propranolol	$C_{16}H_{21}NO_2$	259.35	9.42	3.48 [k]			7.98×10^{-13} [k]			61.7	5.093×10^{-6}
(+)-Levobunolol	$C_{17}H_{25}NO_3$	291.39		2.40 [l]			1.28×10^{-14} [l]			6.933×10^{-7} [l]	
D_6-2,4-D	$C_8H_6Cl_2O_3$	226.04	2.98±0.20	2.598±0.276	139-140.5	345.6±27.0		162.8±42.7	62.24±3.0	900 [e]	
D_4-Nicotinic acid -ethyl ester	$C_8H_9NO_2$	155.16			223-224 [f]			93 [f]			
D_{10}-Carbamazepine	$C_{15}H_{12}N_2O$	246.27	14 [a]	2.25 [a]	190-192	411.0±35.0	1.08×10^{-10} [d]	202.4±46.7	66.34±3.0	17.66 [a]	2.45×10^{-2} [a]

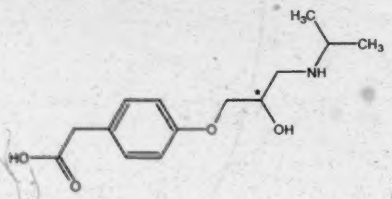
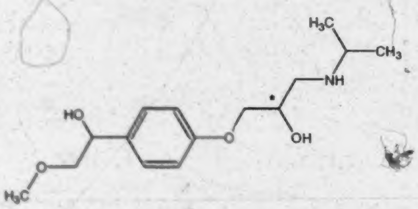
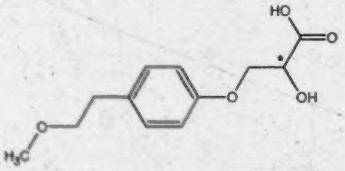
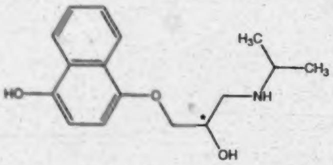
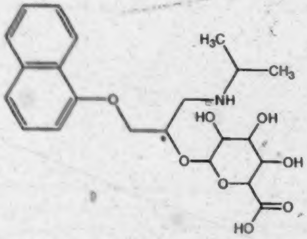
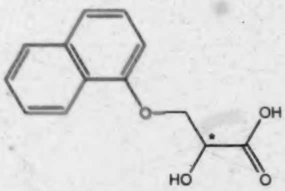
Captions for Table 2.1 Physical Properties: a = (Scheytt et al. 2005), b = (Gardinali et al. 2002), c =(Ternes et al. 2005), d =(Tixier et al. 2003), e = (Kwan et al. 2004), f=(Chemexper.com 2005) , g = (Regan et al. 2005), h = (Loffler et al. 2005), i = (Emblidge et al. 2005), j = (Spectrum 2005), k = (Kostis et al. 1984), l = (Database 2005)

Fig. 2.2 Metabolite Structures

*** marks the chiral center**

Parent Drug	Metabolite Structure	Name
Ibuprofen		2-hydroxyibuprofen
		carboxyibuprofen
Ketoprofen		3-acetylbenzophenone
		2-(3-carboxyphenyl)-propionic acid
Gemfibrozil		hydroxy-metabolite
		hydroxy-metabolite
		hydroxy-metabolite
		carboxy-metabolite

Parent Drug	Metabolite Structure	Name
Fluoxetine		norfluoxetine
Naproxen		O-desmethylnaproxen
Indomethacin		desmethylindomethacin
		desbenzoylindomethacin
		desmethyldesbenzoyl-indomethacin
Caffeine		paraxanthine
Diclofenac		diclofenac glucuronide
Fenoprofen		fenoprofen glucuronide
		4'-hydroxyfenoprofen glucuronide

Parent Drug	Metabolite Structure	Name
Metoprolol		O-demethylmetoprolol
		α-hydroxymetoprolol
		N-dealkylmetoprolol
Propranolol		4-hydroxypropranolol
		Propranolol glucuronide
		Naphthoxylactic acid

2.2.1.3 Naproxen is a creamy white crystalline powder belonging to the arylacetic acid group of the NSAID family. Naproxen is sold by prescription only, and is available in tablet form under the names Naprosyn™, Naprelan™, Aleve™ and Anaprox™. It has anti-inflammatory, analgesic and anti-pyretic activity and is used in both human and veterinary applications at quantities reported between 1.9 to 2.6 tons/yr (Damiani et al. 2002; Kosjek et al. 2005; Metcalfe et al. 2003a). The *S*-(+) isomer has an optical rotatory power of +66.0° with 589.3 nm light at 1g/100mL concentration in chloroform (Milne 2000). Although naproxen is chiral, only the *S*-(+) enantiomer shows therapeutic effect and is the only stereoisomer present in pharmaceutical preparations (Damiani et al. 2002; Hamon et al. 1995).

The active drug works by inhibiting prostaglandin synthesis (Skidmore-Roth 2003). The sodium salt is used because it is absorbed more quickly than the non-salt form. This leads to higher overall blood concentrations after each dose. The specific formulation used in Naprelan™ contains about 30% of the dose in an immediate release form, while the remaining dose is a delayed-release form involving coated microparticles. This results in a peak blood level that occurs after 5 hours, although naproxen can be detected after about 30 minutes. Both the immediate release and delayed release forms have half-lives of about 15 hours, while steady-state concentrations are found after about 3 days. This is consistent with the accumulation of naproxen found in the blood.

Naproxen has an *in vivo* bioavailability of 95% after being absorbed completely from the gastrointestinal tract (Skidmore-Roth 2003; www.rxlist.com 2003). In the first 4-6 hours after ingestion, the tablet disintegrates in the stomach and the extended release microparticles are dispersed to the small and large intestines, a movement that has been confirmed using imaging of healthy volunteers. This disintegration and dispersal phase is considered to be the absorption phase. The extended release microparticles are absorbed more slowly than the regular and immediate release particles. This allows the drug to be taken once a day because the particles maintain the blood levels of naproxen (www.rxlist.com 2003). The presence of food causes a slight decrease in the rate at which naproxen is absorbed in both the regular and extended release formulations of naproxen (www.rxlist.com 2003).

In the body, naproxen is metabolized to 6-O-desmethyl-naproxen. Neither the parent nor metabolite is able to induce metabolizing enzymes (www.rxlist.com 2003). Naproxen is excreted in the urine, with 66-92% of the dose primarily as glucuronide and other conjugates (www.rxlist.com 2003). Unconjugated parent and 6-O-desmethyl-naproxen both account for <1% each and <5% of the drug is excreted in the feces (www.rxlist.com 2003). Metabolites have been found to accumulate in patients with kidney failure since the rate of clearance from the blood is closely related to the rate of excretion.

2.2.1.4 Indomethacin is a member of the NSAID family available in pill, liquid and suppository forms under the name Indocin™. Indomethacin is an effective anti-inflammatory and works well for rheumatoid arthritis, ankylosing spondylitis (rheumatoid arthritis of the spine) and osteoarthritis. It is also used for the treatment of inflammatory processes related to infectious diseases in veterinary medicine (Novakova et al. 2005). Indomethacin can be administered orally, intravenously, in suppository form or as a topical gel (Novakova et al. 2005). It is only useful in terms of symptomatic relief, since it does not halt the course of the diseases. However, patients with rheumatoid arthritis have seen general improvements in terms of reduction of joint swelling, the average number of joints involved and increased mobility. Indomethacin is also effective in treating gouty arthritis.

Peak blood concentrations of indomethacin occur about 2 hours after ingestion of a single dose. Almost the entire dose is bioavailable, with 90% absorbed within four hours. In the body, indomethacin has a mean half-life of 2 hours and is excreted in the urine, in the bile and through metabolism (Lilley et al. 2001). Indomethacin is metabolized to form desmethyl, desbenzoyl and desmethyl-desbenzoyl metabolites all of which, along with the parent, are excreted in an unconjugated form (Novakova et al. 2005; www.rxlist.com 2003). The urine contains about 60% of the dose as parent and metabolites while 33% is found in the feces (www.rxlist.com 2003). Of the 60% found in urine, 26% is found as indomethacin and its glucuronide while of the 26% in feces, 1.5% is indomethacin (Rxlist 26 July 2005).

2.2.1.5 Fenoprofen is a white crystalline powder that is an arylacetic acid derivative member of the NSAID family. Fenoprofen is available by prescription only and is sold under the trade name Nalfon™. In the treatment of rheumatoid and osteoarthritis, fenoprofen has shown effectiveness comparable to that of aspirin and has shown less frequent tinnitus and gastrointestinal reactions such as nausea and dyspepsia than in aspirin treatments. Fenoprofen is chiral and marketed as a racemate.

Because of an anionic polar head group and aromatic lipophilic tail group (Fig. 2.1), fenoprofen is a surface-active drug able to form micelles, which are an aggregate of surfactant molecules (Rades et al. 1998). Liposomes, which contain phospholipid bilayers, can be used as drug carriers to improve the therapeutic effects and decrease the toxicity of the pharmaceuticals (Lopes et al. 2004). Due to the nature of liposomes, they can incorporate hydrophobic molecules into the phospholipid bilayer and hydrophilic molecules into the aqueous phase (Lopes et al. 2004). Amphiphilic compounds, such as many drugs, can self-aggregate to form micelles above their critical micelle concentration (Lopes et al. 2004).

Fenoprofen has a half-life of about 3 hours in the blood, with peak blood levels about 2 hours after an oral dose. Fenoprofen is metabolized to two main metabolites, fenoprofen glucuronide and 4'-hydroxyfenoprofen glucuronide (Rxlist 26 July 2005) which are primarily excreted in the urine. Most (90%) of the dose is eliminated within 24 hours. Albumin, a liver protein, is known to bind 99% of a fenoprofen dose (Rxlist 26 July 2005). Fenoprofen has shown enantioselective binding to albumin in human, rats and rabbits, with selectivity factors (α) of 1.51, 1.24 and 1.22, respectively (Massolini et al. 1993).

2.2.1.6 Ketoprofen is a fine to granular white powder that belongs to the 2-arylpropionic acid group of the NSAID family (Lees et al. 2003). It is available in tablet form by prescription under such trade names as Orudis™ and Oruvail™. Ketoprofen has shown effectiveness in the treatment of rheumatoid and osteoarthritis comparable to that of aspirin, ibuprofen, naproxen, diclofenac and indomethacin.

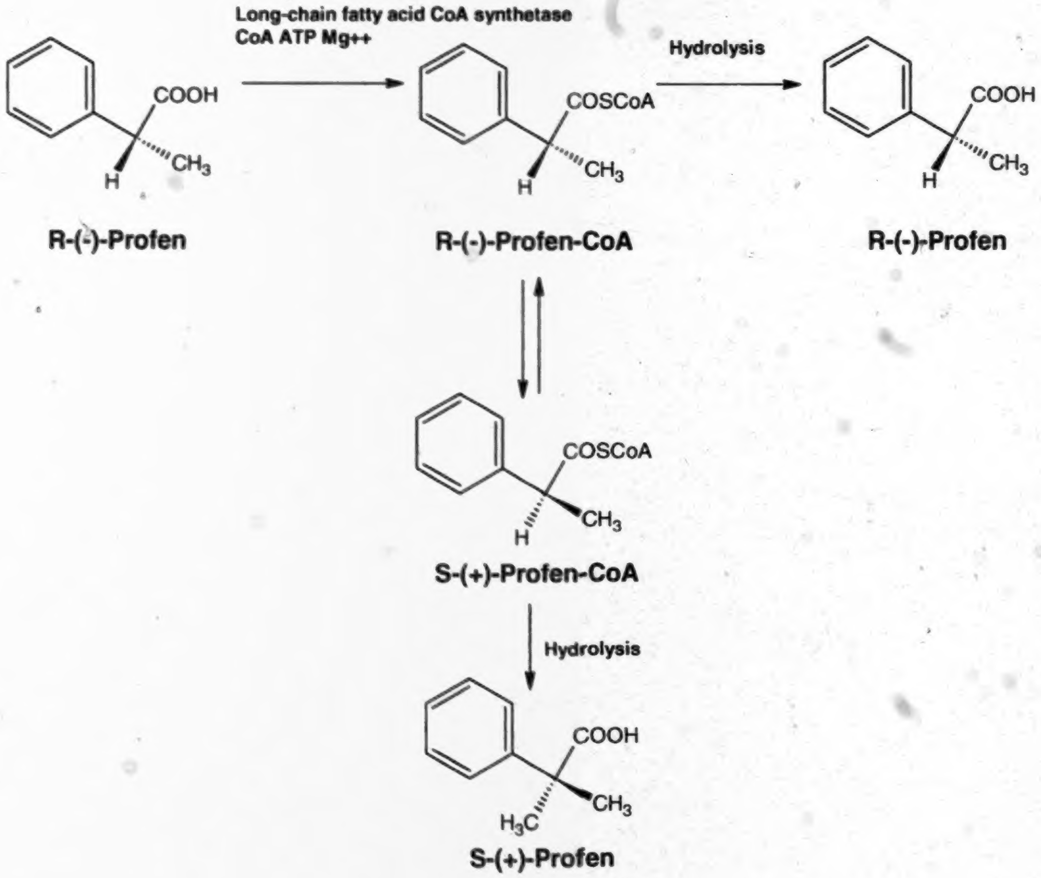
The two brand name forms of ketoprofen, Orudis™ and Oruvail™, only differ in the manner in which the drug is released. Orudis™ targets the stomach, while Oruvail™ is designed to release small amounts of the drug in the small intestine while resisting dissolution in the low pH of the gastric fluid. Ketoprofen is metabolized by the liver and excreted from the kidneys, with a half-life of 2-4 hours (Skidmore-Roth 2003). Within 24 hours of an Oruvail™ dose, 80% of the ketoprofen dose is excreted in the urine (Rxlist 26 July 2005). In the blood, >99% of ketoprofen is bound to plasma proteins, mostly to albumin (Lilley et al. 2001; Rxlist 26 July 2005). Ketoprofen is well absorbed, with peak blood levels found within 2 hours (Skidmore-Roth 2003). Food interferes with the rate of absorption but it does not decrease the total bioavailability (Rxlist 26 July 2005; Skidmore-Roth 2003). Within 24 hours of taking ketoprofen, steady-state concentrations are achieved and the drug does not seem to accumulate in the body (Rxlist 26 July 2005).

In a topical gel formulation, ketoprofen degrades to form small amounts of 3-acetylbenzophenone and 2-(3-carboxyphenyl) propionic acid (Fig. 2.2) after long-term storage (Dvorak et al. 2004). Ketoprofen is metabolized to form an unstable acyl-glucuronide through its conjugation to glucuronide. Because of the instability, it can be easily converted back to the parent compound. Such easy interconversion may allow the metabolite to act as a drug reservoir for the parent drug, which may be important in patients with kidney problems. This reservoir would allow metabolite to accumulate in the blood serum at which time it would then be converted back to active parent drug. Healthy adults who have taken ketoprofen generally have trace amounts of the conjugate but elderly patients have higher amounts, likely due to decreased renal clearance. Ketoprofen does not have any known active metabolites and has not been shown to induce drug-metabolizing enzymes.

Ketoprofen is a chiral drug consisting of *R*-(-) and *S*-(+) enantiomers. Different plasma concentration vs. time profiles are seen for the enantiomers and do not depend on the enantiomeric composition of the solution injected (i.e., whether it is racemic or single enantiomer) (Lees et al. 2003). It is unknown exactly what causes this difference, but it may be due to varying volumes of distribution, elimination rates or plasma protein binding (Lees et al. 2003). There is a chiral inversion mechanism seen for many 2-arylpropionates, including ketoprofen, which converts the less active *R*-(-) enantiomer to the more potent *S*-(+) enantiomer (Castro et al. 2000). Liver enzymes mediate this unidirectional conversion, but it has been described in other organs, such as intestine, kidney and lungs (Castro et al. 2000; Lees et al. 2003). Castro et al. (2000) describe the three steps involved in the chiral inversion mechanism (Fig. 2.3): (1) R-(-)-profen is activated by the formation of acyl-coenzyme A thioester; (2) R-(-)-thioester is converted to S-(+) or is hydrolysed to regenerate the R-(-)-enantiomer; (3) S-(+)-thioester is hydrolysed to end the process (Castro et al. 2000).

Pharmacokinetic enantioselectivity cannot be easily interchanged among species (Lees et al. 2003). When a chiral drug is distributed with both enantiomers, the product actually contains two separate drugs that may not act in the same therapeutic manner. As such, measurements made as to the effectiveness of the drug should take this difference into consideration (Lees et al. 2003). In a study investigating the pharmacokinetics of ketoprofen on the cat, *S*-(+)-ketoprofen was always found at higher levels in the blood than was *R*-(-)-ketoprofen (Lees et al. 2003). *R*-(-)-ketoprofen was cleared from the body nearly five times faster than *S*-(+)-ketoprofen (Lees et al. 2003). These findings agree with earlier studies investigating rats, dogs, horses, monkeys and rabbits (Lees et al. 2003).

Fig. 2.3 Profen Chiral Inversion


Long-chain fatty acid CoA synthetase
CoA ATP Mg++
Hydrolysis
COOH
CH3
H
R-(-)-Profen
COSCoA
CH3
H
R-(-)-Profen-CoA
COOH
CH3
H
R-(-)-Profen
COSCoA
CH3
H
S-(+)-Profen-CoA
Hydrolysis
COOH
CH3
H3C
S-(+)-Profen

Conversely, the enantiomers are nearly racemic in the blood of humans and calves and *R*-(-) is found in higher concentrations in goats and sheep (Lees et al. 2003).The activity of 2-arylpropionates is primarily in the *S*-(+) enantiomer, and although the *R*-(-) enantiomer might be inactive, it could act as a "pro-drug" that would be converted to the active *S*-(+) form through the body's natural inversion process (Lees et al. 2003). This inversion would then increase the dose of *S*-(+)-ketoprofen a patient receives (Lees et al. 2003). Lee et al. (2003) suggest that the formulation of a drug containing the *S*-(+) isomer only would be able to reduce the dose and metabolic load imposed on the body and could possibly reduce species-dependant differences by eliminating inversion pathways (Lees et al. 2003).

2.2.2 Lipid Regulators

Lipid-regulating agents, used in the treatment of hypercholesterolemia, are the leading therapeutic group with worldwide sales of US $26.1 billion in 2003 (Hernando et al. 2004). Lipid regulators work by inhibiting the release of lipoproteins by the liver, increasing the action of lipoprotein lipase and increasing the fecal excretion of neutral sterols. Sterols are the unsaturated solid alcohols of the steroid group, such as cholesterol, and are found in the fatty tissues of plants and animals. Most fats in the body exist as triglycerides, the esters of three fatty acids and glycerol. A high level of low-density lipoprotein (LDL) cholesterol and low level of high-density lipoprotein (HDL) cholesterol is associated with coronary heart disease and is often found in patients with elevated triglyceride levels. Although they are not regulated for such use, some lipid regulators such as clofibrate and gemfibrozil are known to be effective in the reduction of triglycerides and are able to reduce LDL cholesterol levels. This increase in the excretion of sterols could be the reason clofibric acid, a metabolite of clofibrate, is able to lower cholesterol.

Two lipid regulators are included in the list of target compounds (Fig. 2.1, Table 2.1) investigated in this thesis: gemfibrozil and clofibric acid. Each drug will be discussed in detail as follows.

2.2.2.1 Gemfibrozil is a white powder used as a lipid-regulating agent. It is available in pill form under various trade names such as Valeric Acid™, Lopid™ and Lopizid™, and has been found to reduce the occurrence of coronary heart disease by 34%. It is one of the most widely used drugs in its class, and has been shown to lower plasma triglycerides, lower low density lipoproteins and increase high density lipoproteins (Mimeault et al. 2005).

Gemfibrozil works by blocking peripheral lipolysis, and is involved in the reduction of free fatty acids formed during hepatic extraction (Miao et al. 2002). It also reduces blood levels of triglycerides and the very low-density lipoprotein (VLDL) cholesterol that tends to carry them (http://www.reutershealth.com/well connected/doc23.html 15 Aug 2005; Miao et al. 2002). Gemfibrozil is also able to increase the levels of HDL cholesterol as well as its subfractions HDL_2 and HDL_3 and the apoliproteins AI and AII (Miao et al. 2002). The body uses HDL to carry cholesterol to the liver so it can be metabolized and eliminated from the body in the bile. LDL is produced by the metabolism of VLDL (Lee et al. 2005). Gemfibrozil is absorbed from the gastrointestinal tract quite readily with peak blood concentrations occurring 1-2 hours after ingestion (Miao et al. 2002). With long-term use, the half-life of gemfibrozil is 1.5 days (Miao et al. 2002). Gemfibrozil does not accumulate in the body, although concentration is proportional to dosing level (Miao et al. 2002).

Gemfibrozil is metabolized in the liver to form three hydroxyl- and one carboxyl- metabolites (Fig. 2.2) through the oxidation of the ring methyl groups (Miao et al. 2002; Mimeault et al. 2005). These Phase I metabolites undergo further reactions to form five reactive acyl glucuronide conjugates with glucuronic acid. These reactive conjugates may undergo hydrolysis, isomerization, covalent binding and many other typical reactions within the body. Gemfibrozil is mainly excreted from the kidneys, with 60-70% of a dose being excreted in the urine and only 6% in the feces (Mimeault et al. 2005). Gemfibrozil inhibits in vitro testosterone production in rodents and can be concentrated in the blood of goldfish after uptake through the gills (Mimeault et al. 2005). It has a half-life of 19 hours in goldfish plasma, compared to 1.5 hours in human plasma (Mimeault et al. 2005). The kidneys are the main route of excretion in humans (Mimeault et al. 2005). Gemfibrozil has the potential to cause negative effects in goldfish since the fish have the same receptor as humans, the peroxisome proliferator-activated receptor alpha (Mimeault et al. 2005). This is supported by Mimeault et al. (2005), who report unexpected endocrine responses in the form of a 50%

decrease in goldfish testosterone levels after application of 1.5 µg/L gemfibrozil for 14 days.

2.2.2.2 Clofibric Acid is the active metabolite of the drugs clofibrate, etofibrate and etofyllinclofibrate and is used as a lipid regulator to lower cholesterol after its hydrolysis in the body. It is sold by prescription only under the trade name Atromid-*S*™. Clofibrate and gemfibrozil were the only two fibric acid antilipidemics approved for use in the United States until recently, when fenofibrate and benzafibrate were added (Lilley et al. 2001). Clofibric acid and other lipid-regulating drugs are among the highest selling drugs in the world, with $26.1 billion worth of sales worldwide in 2003 (Hernando et al. 2004). In Germany alone, more than 30 tons of clofibrate, etofibrate and etofyllinclofibrate were dispensed in 1992 (Emblidge et al. 2005). As a result of such high sales, and a resistance to degradation, clofibric acid is a very commonly reported drug in open waters, and is estimated to remain in the environment for 21 years (Emblidge et al. 2005). Clofibric acid is a structural isomer of the herbicide mecoprop, and has also been classified as a plant growth regulator but has a higher environmental persistence than mecoprop (Buser et al. 1998c; Emblidge et al. 2005).

Clofibric acid reduces low-density lipoprotein and triglycerides concentrations, although the exact mode of action is not definitively established (Emblidge et al. 2005; Lilley et al. 2001). Clofibric acid generally has a half-life in the body of 18 to 22 hours, but these values can vary by up to 7 hours depending on the patient (Rxlist 26 July 2005). A peak blood plasma concentration of

~200 µg/mL is found in patients taking one dose of clofibrate every twelve hours, with an average concentration of 120 to 125 µg/mL (Rxlist 26 July 2005). When the body hydrolyzes clofibrate, 95% to 99% of the dose is excreted in the urine as either free or conjugated clofibric acid (Rxlist 26 July 2005).

Emblidge and DeLorenzo (2005) investigated the effects of clofibric acid on estuarian species in the belief that long-term, low-level exposure to pharmaceuticals may have non-lethal effects. Reductions have been seen in triacyl glycerides and cholesterol in *Pachygraspus marmoratusa*, a type of crab, after four injections of 50 mg/kg of clofibric acid (Lautier et al. 1986). A study involving catfish (*Ictalurus punctatus*) showed haemorrhaging in the fins, with death resulting after 24 hours are 225 mg/L (Perkins et al. 1998). However, environmentally relevant concentrations of clofibric acid did not affect growth rate in a phytoplankton species (*Dunaliella tertiolecta*), and did not cause changes in protein or cholesterol levels in grass shrimp (*Palaemonetes pugio*) (Emblidge et al. 2005). The mummichog (*Fundulus heteroclitus*), also did not exhibit any changes in protein, cholesterol or lipid levels on exposure to environmentally relevant concentrations of clofibric acid (Emblidge et al. 2005). The authors concluded that the given experimental conditions produced no adverse effects; however, long term effects such as reproductive toxicity, mixture toxicity, bioaccumulation and trophic transfer were not addressed and should be considered (Emblidge et al. 2005).

2.2.3 Stimulants

2.2.3.1 Caffeine is a white powder that belongs to the methylxanthine group of drugs and is commonly used as a stimulant (Horrigan et al. 2004). In addition to its diuretic, respiratory, cardiovascular and central stimulant properties, caffeine has been used therapeutically to treat narcolepsy, asthma and apnea (Daly et al. 1998). It is isolated in pure form during the decaffeination of coffee. It is an alkaloid that naturally occurs in more than sixty species of plants such as coffee, cacao and cola tree seeds and tea tree leaves (Buerge et al. 2003). The main sources of consumption are coffee, tea and caffeinated cola drinks (Daly et al. 1998). It has one of the highest consumption rates of all drugs in the world, with an estimated 70 mg per person per day consumed throughout the world (Buerge et al. 2003; Daly et al. 1998). Because of such high annual usage, it is not surprising it has been detected in wastewater, surface water and ground water throughout the world (Buerge et al. 2003; Gagne et al. 2005; Gardinali et al. 2002; Glassmeyer et al. 2005). Caffeine has been used as a tracer for human domestic waste since it is found in almost all waters that have been contaminated by human waste (Glassmeyer et al. 2005; Weigel et al. 2004a).

The effects of caffeine in humans are dose dependant (Daly et al. 1998; Wurts et al. 2000). Low doses cause central stimulation that can affect the cardiac, cerebral and respiratory systems in the body while high doses cause negative side effects such as anxiety, restlessness, sleeplessness, tension, nervousness, psychomotor agitation and has effects on bone status and calcium balance, cardiovascular effects, behavioural changes, increased incidences of cancer and effects on male fertility (Buerge et al. 2003; Daly et al. 1998; Knight et al. 2004; Wurts et al. 2000). Additionally, caffeine can increase the effect of certain analgesics, like acetaminophen, and act as a diuretic (Horrigan et al. 2004). Both humans and animals exhibit caffeine withdrawal symptoms (Daly et al. 1998). Animals show decreased locomotor activity and operant-responding, while human symptoms include headache, fatigue, apathy and drowsiness (Daly et al. 1998). Maximum withdrawal symptoms appear after 1-2 days but are over within a few days and can be easily relieved by taking caffeine (Daly et al. 1998).

At the dose of caffeine typically consumed by humans, caffeine works by blocking adenosine receptors (Daly et al. 1998; Lorist et al. 2003). Blocking can even be seen at the low concentrations found after a single cup of coffee (Lorist et al. 2003). Synergistic effects may be seen in which other drugs may elevate adenosine levels, allowing caffeine to work in different pathways than is seen without the drugs being present (Daly et al. 1998). Other mechanisms of action include inhibition of phosphodiesterase and mobilization of intracellular calcium, but these require higher concentrations of caffeine than are likely reached from dietary sources (Lorist et al. 2003).

Caffeine is almost completely absorbed (99%) from the gastrointestinal tract into the bloodstream after oral ingestion, with peak plasma levels occurring 30-60 minutes after ingestion (Lorist et al. 2003). In the body, caffeine is metabolized to its major metabolite, paraxanthine, with an estimated 80% efficiency (Horrigan et al. 2004). Despite the high percentage of metabolism and the large amount of caffeine ingested worldwide, the metabolite's effects have not been investigated (Metcalfe et al. 2003b). Caffeine can be found in the urine but only about 3% remains unmetabolized (Metcalfe et al. 2003b). Caffeine is able to cross all biological membranes, including the blood-brain barrier and the placental barrier and is distributed throughout the body (Lorist et al. 2003). The approximate half-life of caffeine is 3-5 hours although drugs such as nicotine and oral contraceptives may affect this rate (Lorist et al. 2003).

2.2.4 Selective Serotonin Re-Uptake Inhibitors (SSRI)

Selective Serotonin Re-Uptake Inhibitors (SSRI) inhibit serotonin (5-hydroxytryptamine, 5-HT) reuptake at presynaptic neuronal membranes (Brooks et al. 2003). Serotonin is a neurotransmitter involved in the modulation of many behavioural and physiological functions and is known to modulate mood, emotion, sleep and appetite. By blocking the uptake of serotonin, SSRI increase serintonin concentrations in the body and are therefore useful as antidepressants. The extent to which SSRIs are specific is somewhat debatable, with some authors reporting they bind to norepinephrine uptake and Sigma receptors, and some claiming they do not (Brooks et al. 2003; Sabbioni et al. 2004). Norepinephrine is responsible for increased heart rate and blood pressure as a response to short-term stress (Lilley et al. 2001). Binding of these neurotransmitters should cause a reduction in these sympathetic nervous system responses, although the consequence of such binding is unknown. Fluoxetine has shown little effect on muscarinic, histaminic, serotonergic and noradrenergic receptors (Sabbioni et al. 2004). The anticholinergic, sedative and cardiovascular effects of classical tricyclic antidepressants like amitriptyline are associated with these receptors (Rxlist 26 July 2005).

2.2.4.1 Fluoxetine, a white powder, was the first SSRI marketed for the treatment of depression, and has become one of the most highly prescribed anti-depressants since released in 1988 (Lilley et al. 2001). It is sold in pill and solution form under the trade names Prozac™, Reneuron™, Adofen™ and Astrin™ and is prescribed to over 40 million people worldwide annually (Flaherty et al. 2005). Besides depression, fluoxetine has found use in the treatment of obsessive-compulsive disorder, bulimia nervosa and panic fits (Sabbioni et al. 2004). Some of the negative side effects experienced include nausea, insomnia, nervousness and somnolence (Lilley et al. 2001; Sabbioni et al. 2004). Fluoxetine is a racemic mixture of two isomers. The (*S*)-enantiomer is the dominant one seen in blood samples under steady state conditions because it is eliminated more slowly (Shen et al. 2002). Both enantiomers have almost equal pharmacological activity in animal studies and are both potent and specific SSRIs (Rxlist 26 July 2005).

Fluoxetine has a half-life in the body of 1-3 days and reaches a peak concentration after 6-8 hours, but it does not seem to follow linear pharmacokinetics (Lilley et al. 2001; Rxlist 26 July 2005). A slow elimination rate means it can take weeks for the drug to leave the system after discontinuation because the drug can accumulate in the body (Skidmore-Roth 2003). Peak blood concentrations were found in the range of 30.1–134.6 ng/ml after use of

30-75 mg/day (Djordjevic et al. 2005). Fluoxetine exhibits a long elimination half-life of 1-4 days, with an average of 3 days (Djordjevic et al. 2005). Elimination half-life may be further extended if high doses are taken over an extended period of time (Djordjevic et al. 2005). There is the possibility of drug interactions if fluoxetine is taken with other drugs that are also metabolized by cytochrome P450, one of the body's most powerful detoxifying agents. Cytochrome P450 oxidation is the main mechanism by which drugs are metabolized in the body to increase the drug's water solubility.

Fluoxetine undergoes metabolism in the liver by cytochrome P450 isoenzymes to form the active metabolite norfluoxetine, as well as other unidentified metabolites (Fig. 2.2). About 10% of the dose is excreted as unmetabolized parent compound and 7% is excreted as the metabolite (Brooks et al. 2003; Skidmore-Roth 2003). The active metabolite, norfluoxetine, is also a chiral molecule and results from the demethylation of fluoxetine. S-norfluoxetine is also an SSRI, and has a potent and selective activity almost equal to that of *R*- and *S*-fluoxetine while *R*-fluoxetine is not as potent as *S*-fluoxetine or *S*-norfluoxetine (Djordjevic et al. 2005; Rxlist 26 July 2005). Norfluoxetine seems to be eliminated by hepatic metabolism to inactive metabolites and is excreted in the urine (Rxlist 26 July 2005). The half-life of norfluoxetine is 4-16 days for both acute and chronic administrations (Rxlist 26 July 2005). Blood concentrations were 72-258 ng/ml after 30 days and it seems to follow linear pharmacokinetics where metabolism is proportional to dose (Rxlist 26 July 2005). The long half-life combined with the activity of norfluoxetine could help contribute to fluoxetine's long lasting effects.

Fluoxetine has been shown to be lethal to certain aquatic organisms. Freshwater midges (*Chironomus tentans*) had an estimated LC_{50} of 15.2mg/kg while water fleas (*Ceriodaphnia dubia* and *Daphnia magna*) and fathead minnows (*Pimephales promelas*) had average LC_{50} values of 0.756, 2.65 and 2.28 μM, respectively (Brooks et al. 2003). Fluoxetine has shown toxicity in sunfish, with exposure leading to increased plasma serotonin levels (Richards et al. 2004). The increased levels of serotonin constrict the arterio-arterial brachial vasculature, leading to impaired gas exchange and hypoxia, ultimately leading to death (Richards et al. 2004). The sunfish toxicity may be due to synergistic interactions, wherein a combination of drugs increases the potency of one or all, but this needs to be further investigated (Richards et al. 2004). During overdose in humans, no deaths were reported for fluoxetine levels from 232 to 1390 ng/mL after patients taking doses up to 1500 mg (Sabbioni et al. 2004). One patient did die of poisoning, with fluoxetine blood levels of 5600 ng/g and 3300 ng/g of norfluoxetine (Sabbioni et al. 2004).

2.2.5 β-Adrenergic Blocking Drugs (β-Blockers)

β-Adrenergic blocking drugs (β-blockers) are commonly used in the treatment of cardiovascular diseases such as angina pectoris, cardioarrythmia, hypertension and myocardial infarction. In addition to their anti-hypertensive properties, this class of drugs has also found use for non-cardiovascular indications including migraine prophylaxis and anxiety disorders. Some β-blockers are used in eye drops for the treatment of glaucoma and acular hypertension. Because of their excellent efficacy and safety records, β-blockers are one of the most commonly prescribed classes of drugs used in the treatment of cardiovascular diseases (Kostis et al. 1984). However, they can be very toxic and have a narrow therapeutic range, requiring a relatively high dose (5-100 mg) (Kataoka et al. 1999). β-blockers competitively inhibit access to β-adrenoceptors by β-agonists such as epinephrine and norepinephrine (Kostis et al. 1984). Some β-blockers such as metoprolol and atenolol exhibit cardioselectivity only at low doses, while others such as propranolol are non-cardioselective β-blockers. Although both selective and non-selective β-blockers are commonly prescribed, patients with certain medical conditions such as asthma have shown contraindications when using non-selective β-blockers. Some of the more common side effects include bradycardia, fatigue, cold limbs and sleep disturbances (Cruickshank et al. 1994).

Three β-blockers are included and discussed in the list of target compounds (Fig. 2.1, Table 2.1) investigated in this thesis: metoprolol, atenolol and propranolol.

2.2.5.1 Metoprolol is a white crystalline powder used as a β-blocker. It is available by prescription in tablet and injection formulations under the trade names Lopressor™, Betaloc™ and Toprol XL™. Both tartrate and succinate salts are available. Metoprolol is a cardioselective β-blocker and blocks stimulation of β_1-adrenergic receptors within vascular smooth muscles

(Cruickshank et al. 1994; Skidmore-Roth 2003). It decreases blood pressure and heart rate, and reduces the oxygen requirements of the heart. Metoprolol is chiral and is marketed as the racemate.

All metoprolol formulations are well absorbed from the gastrointestinal tract. Peak blood levels are seen 20 minutes after intravenous injection and 2-4 hours after ingestion of tablet formulations (Skidmore-Roth 2003). Food does not cause a significant delay in absorption of the drug but can cause a 50% increase in the bioavailability of metoprolol. Despite complete absorption, only about 50% of a single therapeutic dose will reach the circulatory system due to first-pass metabolism (Cruickshank et al. 1994). Bioavailability is dose-dependent after a single dose but increases with chronic therapy (Cruickshank et al. 1994).

Metoprolol is extensively metabolized in the liver, with a half-life of 3-4 hours (Skidmore-Roth 2003). Due to this short half-life, metoprolol generally needs to be given in divided doses (Cruickshank et al. 1994). Within 24 hours of oral administration, 90% of a labelled metoprolol dose was recovered in human urine (Cruickshank et al. 1994). Of this 90%, only 4% was unmetabolized metoprolol (Cruickshank et al. 1994). Metoprolol is metabolized via oxidative pathways (Fig. 2.2). Two of metoprolol's metabolites, O-demethylmetoprolol and α-hydroxymetoprolol, are active and selective β_1-antagonists. However, they are 5-10 times less potent than the parent drug (Cruickshank et al. 1994). Demethylation to form O-demethylmetoprolol is the major metabolic pathway and accounts for 65% of the dose (Mehvar et al. 2001). The hydroxy metabolite, α-hydroxymetoprolol, occurs at about 50% of the parent drug concentration

(Cruickshank et al. 1994). The other two metabolites are inactive acidic metabolites that make up the majority of metabolite concentration in human plasma (Cruickshank et al. 1994). The hydroxylation pathway is stereoselective for *S*-(-)-metoprolol while the demethylation pathway is stereoselective for *R*-(+)-metoprolol (Mehvar et al. 2001). This is responsible for the stereoselectivity observed for plasma concentrations of metoprolol.

2.2.5.2 Atenolol is a white powder used as a β-blocker. It is available by prescription in tablet and injection formulations under the trade name Tenormin™ and is the most hydrophilic of the available β-blockers. Atenolol is a cardioselective β-blocker and blocks stimulation of β_1-adrenergic receptors within vascular smooth muscles (Cruickshank et al. 1994; Skidmore-Roth 2003). It is commonly used to prevent future heart attacks in patients who have previously had one and in the treatment of hypertension and angina. It decreases blood pressure and heart rate, and reduces the oxygen requirements of the heart. At higher doses, it inhibits β_2-receptors in bronchial systems (Skidmore-Roth 2003). Atenolol is chiral and is marketed as the racemate, although the *S*-(-)-enantiomer is the active drug. The *S*-(-)-enantiomer is 10 times more potent than the *R*-(+)-enantiomer (Davies 1990).

Atenolol is unmetabolized in the human body and is excreted through the kidneys (50%) and feces (50%) as unabsorbed drug following an oral dose (Skidmore-Roth 2003). After an IV dose, 90% of atenolol is excreted in the urine and 10% in the feces (Cruickshank et al. 1994). Atenolol is only partially absorbed (50-60%) after oral administration and peak plasma concentrations are found 2-4 hours after administration (Skidmore-Roth 2003). The half-life of atenolol is 6-7 hours (Skidmore-Roth 2003). Absorption of atenolol occurs in the small intestine, with a large proportion absorbed in the jejunum, which is halfway down the small intestine, below the duodenum (Cruickshank et al. 1994). The plasma concentration of the less active *R*-(+)-isomer is slightly higher (<20%) than that of the *S*-(-)-isomer because the kidneys preferentially clear the active isomer (Mehvar et al. 2001).

Since atenolol is unmetabolized in the human body, 90% of the drug is found unmetabolized in urine (Cruickshank et al. 1994). Ten percent or less is metabolized to form a hydroxylated metabolite and a glucuronide conjugate. However, both metabolic pathways are minor. Hydroxyatenolol shows weak activity, with potency only about 10% that of atenolol (Cruickshank et al. 1994). Atenolol shows no pharmacokinetic or pharmacodynamic interactions between the two enantiomers (Mehvar et al. 2001). *S*-(-)-atenolol given at half the dose of racemic atenolol resulted in the same effects as racemic atenolol (Mehvar et al. 2001).

2.2.5.3 Propranolol is a white crystalline solid used as a β-blocker. It is available by prescription only in tablet and injection formulations under the trade names Detensol™ and Inderal™. Propranolol is a non-specific β_1- and β_2-blocking agent and was the first β-blockers introduced, in 1965 (Davies 1990; Lilley et al. 2001). It is commonly used to prevent future heart attacks in patients who have previously had one and in the treatment of hypertension and angina. It has found additional use in the treatment of such conditions as migraine headaches, anxiety and essential tremors. At higher doses, it inhibits β_2-receptors in bronchial systems (Skidmore-Roth 2003). Propranolol is a highly lipophilic chiral drug and is marketed as the racemate, although the *S*-(-)-enantiomer is the active drug. The *S*-(-)-enantiomer is 100 times more potent than the *R*-(+)-enantiomer (Davies 1990).

Propranolol is well absorbed and extensively metabolized in the liver. First-pass metabolism in the liver determines the amount of propranolol present in the circulatory system. As a result, only about 30% of a propranolol dose is bioavailable. Dose dependence is seen for propranolol, and a progressively higher fraction of the dose reaches the circulatory system as the dose increases. Chronic treatment is known to increase the available fraction of a dose. When given with food, propranolol blood levels increased two-fold, as compared to given in a fasting state (Cruickshank et al. 1994). The half-life of propranolol is 3-5 hours and peak plasma concentrations are found 1-1.5 hours after administration (Skidmore-Roth 2003). Pharmacokinetic and pharmacodynamic interactions have been seen between the enantiomers of propranolol. The less active *R*-(+)-propranolol tends to show lower plasma concentrations when administered as a pure enantiomer than when given as the racemate (Mehvar et al. 2001). However, the more active *S*-(-)-propranolol shows the same kinetics whether administered as single enantiomer or as racemate (Mehvar et al. 2001).

Propranolol is metabolized through glucuronidation, ring hydroxylation and side chain oxidation (Mehvar et al. 2001). It forms a number of metabolites, including 4-hydroxypropranolol, propranolol glucuronide and the major metabolite, naphthoxylactic acid (Fig. 2.2) (Cruickshank et al. 1994). However, only 4-hydroxypropranolol shows β-blocking activity in animal studies (Cruickshank et al. 1994). It is almost as potent a β-blocker as propranolol in animal studies, but has a shorter half-life. Naphthoxylactic acid accounts for 25-65% of the dose in urine, while 4-hydroxypropranolol accounts for 20-30% of the dose in urine (Cruickshank et al. 1994). The ring hydroxylation shows a substrate stereoselectivity for *R*-(+)-propranolol (Mehvar et al. 2001). When 4-hydroxypropranolol is further conjugated by glucuronic acid, the *S*-(-)-enantiomer is favored but the *R*-(+)-enantiomer is favoured during conjugation to sulfate (Mehvar et al. 2001). Substrate concentrations affect the enantioselective dealkylation of propranolol. At low concentrations, the *R*-(+)-enantiomer is preferentially metabolized while the *S*-(-)-enantiomer is preferentially metabolized at high concentrations (Mehvar et al. 2001). Glucuronide formation favours the *S*-(-)-propranolol (Mehvar et al. 2001). Overall, higher plasma concentrations of the *S*-(-)-enantiomer are seen in humans because the metabolism of propranolol is stereoselective for the less active *R*-(+)-enantiomer (Mehvar et al. 2001).

Chapter 2.3 Environmental Fate Processes and Occurrence

2.3.1 Environmental Fate Processes

2.3.1.1 Air-Water Partitioning

Henry's Law describes the partitioning of volatile organic chemicals between atmosphere and aqueous solution (Crosby 1998). It states that the equilibrium concentration of analyte in the vapour phase is proportional to that in the aqueous phase:

$$H' = C_{air}/C_{water} \quad \text{(dimensionless)} \qquad (2.3.1)$$

$$K_H = P/C_{water} \quad \text{(Pa m}^3\text{/mol)} \qquad (2.3.2)$$

$$H = K_H/RT \qquad (2.3.3)$$

where C_{air} is the concentration of analyte in air, C_{water} is concentration in water and P is the vapour pressure. Thus, a compound with a higher Henry's Law constant (K_H) has a higher volatility than one with a lower K_H. As can be seen in Table 2.1, all of the target compounds have K_H values on the order of 10^{-7} atm m^3 mol^{-1} or less, indicating they are not likely to escape from the aqueous phase. Their occurrence in the environment will likely be from processes such as sorption onto solids or dissolution into aqueous phases instead of volatilization from aqueous solutions. Partitioning between air and water layers is only possible for unionized compounds, so C_{water} in the above equations refers only to the concentration of compound that is unionized in water. Target analyte pKas are shown in Table 2.1 and are all < 5, with the exceptions of fluoxetine and carbamazepine. This indicates the acidic target drugs will be largely ionized at typical environmental pHs of 5.5 to 8.

2.3.1.2 Sorption

The fate of pharmaceuticals in the environment can be affected by sorption processes (Drillia et al. 2005; Scheytt et al. 2005; Schwarzenbach et al. 2003). Adsorption is the attachment of an analyte onto the two-dimensional surface of a particle while absorption is the sorption into the three-dimensional matrix of a particle (Schwarzenbach et al. 2003). Adsorption depends on particle properties like organic matter content and pH. The presence of colloidal matter and dissolved organic carbon (DOC) has been shown to increase the mobility of highly sorptive compounds and environmental pH can affect the speciation of analyte molecules (Kay et al. 2005). Analyte properties including water solubility and octanol-water partition coefficient (K_{OW}, for the neutral form of the analyte) are also important in determining the extent of sorption (Drillia et al. 2005). Lower water solubility generally gives higher binding potentials, although water solubility increases above the pKa due to ionization of the compound (Crosby 1998; Kenawi et al. 2005). Hydrophobicity is measured by the K_{OW} and must be considered when investigating the environmental fate of organic compounds. A hydrophobic compound will have a higher K_{OW} and subsequently higher binding potentials, with a lower proportion of the compound found in natural waters (Crosby 1998).

All of the target pharmaceuticals have log K_{ow} of 4.3 or less (Table 2.1), indicating they are likely to be found in the aqueous layer instead of sorbed to any organic or sediment layers under typical concentrations of organic particles in natural waters. In addition, they are not likely to bioaccumulate (Fisk et al.

1998; Gobas et al. 1999). Given the typical ambient pH in environmental waters, the effective K_{ow} will be lower than expected. The relationship between pH and pKa will be derived as follows:

$$K_{ow} = C_{octanol}/C_{water} \tag{2.3.4}$$

where

$$C_{water} = [HA] = [A^-]/10^{(pH - pKa)} \tag{2.3.5}$$

and

$$K_{ow} = C_{octanol} \times 10^{(pH - pKa)}/ [A^-] \tag{2.3.6}$$

Using ibuprofen (pKa = ~4.5) as an example, at an ambient pH of 6.5 then unionized ibuprofen is present at 100 times lower concentration than will the ionized form. The low concentration of the neutral form will then make both K_{ow} and K_H appear larger than they should be because those equations rely on the aqueous concentration of the compound.

With the exception of caffeine, there is a hundred-fold difference in the K_{OW} of the pharmaceuticals, which could affect the environmental fate of the drugs relative to each other. Caffeine is known to be quite persistent in aqueous samples because it has both high water solubility (13.5 g/L) and a low K_{ow} (Gardinali et al. 2002). Clofibric acid is also known to have a high mobility in natural waters (Buser et al. 1998b). Ibuprofen is considered moderately persistent in natural waters, although Ashton et al. (2004) found ibuprofen in consistently higher concentrations than any of the other pharmaceuticals they studied in sewage treatment plant effluents in the United Kingdom (Ashton et al. 2004).

A sorption isotherm describes the relationship between a compound's concentration in solution and the total sorbate concentration. The Freundlich isotherm is an empirical equation commonly used to fit experimental data:

$$C_{sorb} = K_F \cdot C_w^{1/n} \quad (2.3.7)$$

where C_{sorb} and C_w are the concentration of analyte on the solid and in solution, respectively, K_F is the Freundlich constant and 1/n is a linearity parameter with an empirical value determined by the adsorbent. If the logarithmic form of this equation is taken,

$$\ln C_{sorb} = (1/n) \ln C_w + \ln K_F \quad (2.3.8)$$

values for n and K_F can be calculated using linear regression (Scheytt et al. 2005). For the case of n = 1, the Freundlich isotherm (Eqn. 2.3.4) becomes linear and is known as Nernst partitioning and the proportionality constant becomes the solid-water distribution coefficient, K_d. This distribution coefficient is the combination of numerous sorption mechanisms, including adsorption and absorption to natural organic matter, interactions with mineral surface, interactions of charged species with solids and bonding to several different kinds of surface moieties (Scheytt et al. 2005). Normalizing the sorption coefficient with respect to the organic carbon content (fraction of organic carbon, f_{OC}) gives the organic carbon normalized sorption coefficient K_{OC}:

$$K_{OC} = K_d / f_{OC} \quad (2.3.9)$$

This can be used to reduce the variability of sorption coefficients that results from the variability of soil composition (Crosby 1998). The K_{OC} assumes sorption predominantly occurs to the natural organic matter in the soil or sediment, as opposed to the inorganic carbon found in molecules such as carbonate. Soil is a mixture of clay minerals, sand and silt, organic matter and water. Although the mineral fraction adsorbs analytes to an extent, the organic fraction is the more important adsorbent for neutral molecules. However, most of the target pharmaceuticals investigated here are ionized at typical environmental pHs and are not expected to adsorb to the organic fraction.

Sorption is important for input, transportation and transformation of these active compounds in the aquatic environment, in soils and even in natural sandy sediments (Drillia et al. 2005; Scheytt et al. 2005). Through biological (biodegradation) and physico-chemical processes such as settling and flotation, the interactions between analytes and solid particles aid in analyte removal during the wastewater treatment process (Roberts et al. 2005). Note that the K_{oc} model discussed above is not designed to handle the activated sludge found during wastewater treatment, as the organic carbon found in waste biosolids is different than the organic carbon matter found in soil and sediments. However, environmental fate depends on the type of sediments and organic carbon present, as well as the physicochemical properties of the pharmaceuticals being investigated (Drillia et al. 2005; Scheytt et al. 2005). The amount of drug present in the environment, the amount of rain received in an area and the soil type affect the mobility of pharmaceuticals in the environment and the potential for groundwater contamination and drinking water deterioration (Drillia et al. 2005). A higher environmental concentration means more compound is available for transport within the environment and water-soluble drugs, with low K_{OW}, are therefore sensitive to the amount of rain received in the area. For example, analytes with low adsorption coefficients, such as ibuprofen and clofibric acid, will remain in the aqueous phase (Loffler et al. 2005). Heberer (2002) reported polar pharmaceuticals in ground water and Ternes et al. (2002) found negligible sorption onto sludge for clofibric acid, ibuprofen, indomethacin, ketoprofen and diclofenac, although diclofenac was present in digested sludge at 0.22 µg/g (Heberer 2002; Roberts et al. 2005; Ternes et al. 2005).

Soil has an overall negative charge due to the carboxylic acid moieties on its organic fractions (Crosby 1998; Drillia et al. 2005). Charge on pharmaceuticals is pH dependent, which allows the drugs to interact differentially with the soil depending on its charge. Given that the typical pH range of environmental waters is 5 – 7, acidic pharmaceuticals with pKa > 5 will be negatively charged and thus repelled by the soil. Basic pharmaceuticals with pKa < 7 will be attracted to the soil particles because they will be positively charged (Crosby 1998; Kay et al. 2005). Scheytt et al. (2005) found diclofenac and ibuprofen had higher mobility in soil columns and under natural aquifer conditions than could be expected based on K_{ow} alone. This implies their ionic character competes with the sorption of these compounds, with more dissociation leading to less sorption than predicted. Pharmaceuticals that are resistant to biodegradation may end up in bodies of water through wastewater contamination or in the soil from application of sewage sludge, depending on their ability to sorb onto WWTP sludge (Drillia et al. 2005). There have been some reports, however, that do describe the removal of target pharmaceuticals from the aquatic environment through sedimentation. In a study of the Lake Greifensee water column, Tixier et al. (2003) found ibuprofen was being removed through sedimentation due to the high organic carbon content (2 mg/L) and a high settling rate (1 m/day). Overall, however, sorption onto sediment particles is not believed to be a relevant removal process for the target pharmaceuticals (Buser et al. 1998c; Drillia et al. 2005; Ternes et al. 2005).

2.3.1.3 Bioaccumulation

The partitioning and concentration of aqueous solute into living organisms is known as bioconcentration. It is possible for organic pollutants, including pharmaceuticals, to concentrate within the fat stores of an organism. If body fat is considered to be a "solvent", it is easy to picture the partitioning of a non-ionic compound into this phase from the aqueous phase. The fat of aquatic organisms living in polluted waters is in direct contact with pollutants through their gills and skin and will come to equilibrium with environmental pollutant concentrations. The bioconcentration factor (BCF) describes the ratio of concentration in the organism to that in the water:

$$BCF = C_{organism}/C_{water} \qquad (2.3.10)$$

The BCF will vary with chemical, species and fat content. If the organism is returned to clean water, $C_{organism}$ will decrease as the pollutant is released back into the water through depuration. For bioconcentration in aquatic organisms, exposure is largely due to water and little results from food but for terrestrial organisms, food is the prime exposure route. Bioaccumulation is the sum total of all processes that result in increasing concentration of a chemical in biotic food webs. Bioconcentration and bioaccumulation result in an increased concentration of the pollutant in the organism, and may reach the dose needed for deleterious biological effects (Crosby 1998). Some studies have found diclofenac may be a risk to fish like rainbow trout in effluent-rich waters and have shown concentration-related accumulations in the liver, kidney and gill (Triebskorn et al. 2004). The lowest observed effect concentration for cytological alterations in these organs was 1 µg/L, an environmentally relevant concentration (Triebskorn et al. 2004). Given the relatively high aqueous solubility and low sorption potential of the target pharmaceuticals, they are available in the aquatic environment for uptake into organisms. However, as discussed above, they are not expected to bioconcentrate to a great extent. Interestingly, some SSRIs have been shown to have an effect on zebra mussel spawning, with spawning induced in both sexes at concentrations much lower than that required for serotonin itself (Weigel et al. 2004a). Males required only 5×10^{-8} M of fluoxetine while females required 5×10^{-6} M of fluoxetine to induce a significant percentage of spawning (Weigel et al. 2004a).

2.3.2 Chemical Transformations in the Environment

2.3.2.1 Phototransformation

Pharmaceuticals are susceptible to phototransformation within the environment (Isidori et al. 2005; Tixier et al. 2003). Phototransformation is useful for the degradation of aquatic pollutants (Crosby 1998). Lam et al. (2004) have demonstrated that exposure to sunlight is an important factor in limiting persistence of pharmaceuticals with shorter half-lives. Phototransformation processes are strongly time and site dependent, and the intensity variations caused by differences in both latitude and longitude can strongly influence the rate of photodegradation of pharmaceuticals in surface waters (Andreozzi et al. 2003; Tixier et al. 2003).

There are two types of photolysis, direct and indirect. Direct photolysis results from the direct absorption of sunlight, while indirect photolysis results from reactions with hydroxyl radicals and singlet oxygen generated from natural photosensitizers like nitrate and humic acids (Andreozzi et al. 2003). Direct photolysis has been shown for carbamazepine, diclofenac, fluoxetine, propranolol and naproxen (Buser et al. 1998c; Isidori et al. 2005; Lam et al. 2004; Lam et al. 2005; Tixier et al. 2003; Uwai et al. 2005). Ibuprofen does not absorb light at ambient wavelengths and phototransformation is not believed to be a significant degradation pathway (Tixier et al. 2003). Lam et al. (2005) demonstrated that the direct photolysis degradation of fluoxetine and other trifluoromethylated compounds is through defluorination of the trifluoromethyl group and has a half-life of 7±1 days in pure water exposed to natural sunlight (Lam et al. 2005). In a sunlight simulator, however, the observed pseudo first order degradation rate for fluoxetine in pure water was 0.0126± 0.001 h^{-1}, which corresponds to a half-life of 55.2±3.6 hr (Lam et al. 2005). Although the authors did not explain the differences in the degradation rates, perhaps this difference is due to differences in the energy of the light used in the experiments. Indirect photolysis of fluoxetine through reaction with OH• is faster than direct photolysis, with a bimolecular rate constant between $(8.4\pm0.5)\times10^{9}$ $M^{-1}s^{-1}$ and $(9.6\pm0.8)\times10^{9}$ $M^{-1}s^{-1}$ yielding O-dealkylated compounds (Lam et al. 2005). Carbamazepine has a half-life of about 100 days in winter at 50°N in distilled water (Andreozzi et al. 2003). Indirect photolysis of carbamazepine tends to form epoxides and could contribute to the overall fate. Carbamazepine has a second order rate constant for its reaction with ozone and hydroxyl radicals of 3×10^{5} $M^{-1}s^{-1}$ (Huber et al. 2003; Lam et al. 2004). Carbamazepine had a half-life of 82 days in a microcosm study and of ~1 day in a sunlight simulator (Lam et al. 2004). This same microcosm study found caffeine to be the least persistent with a half-life of ~1 day (Lam et al. 2004). Diclofenac is rapidly degraded when exposed to sunlight and has a half-life of <1 hr (Buser et al. 1998c). Buser et al. (1998) determined photolysis rates of 0.006 to 0.14 day^{-1} in June in Lake Greifensee, and Andreozzi et al. (2003) found a diclofenac half-life of 5 days in winter at 50° N in distilled water. Poiger et al. (2001) determined photolysis rates of 0.3-18 d^{-1} in Lake Greifensee using samples collected during 1998. As a result, photodegradation is the predominant method of elimination of diclofenac in the lake, with an estimated >90% of diclofenac entering the lake being eliminated from the lake (Buser et al. 1998c; Poiger et al. 2001; Tixier et al. 2003). Although little is available on the environmental fate of naproxen, phototransformation appears to be the main elimination process (Isidori et al. 2005; Tixier et al. 2003). Ketoprofen also is believed to be phototransformed in the environment (Tixier et al. 2003). Ibuprofen showed no degradation up to 37 days in sterile water, even when exposed to daylight, although nonsterile conditions showed degradation in both dark and light conditions (Buser et al. 1999). This degradation is most likely not due to phototransformation and may be due to biological processes (Buser et al. 1999). Overall, ibuprofen is not considered to phototransform because it does not absorb sunlight at visible wavelengths (Tixier et al. 2003). Structural differences between the target pharmaceuticals are likely contributors to the differences seen in rates of photodegradation. Larger molecules with more aromatic rings are more likely to absorb visible light and therefore phototransform. This has been seen above for carbamazepine, diclofenac, fluoxetine, naproxen, caffeine and ketoprofen, all of which have two or more aromatic rings. Direct phototransformation was not observed for ibuprofen and clofibric acid, both of which have only one aromatic ring (Tixier et al. 2003). Although data could not be found for gemfibrozil, it is expected to follow this trend and not phototransform, as it contains one aromatic ring. Propranolol is relatively unstable and must be protected from light. The photodegradation products of propranolol are naphthalene, 1,4-naphthoquinone and 6-hydroxy-1,4-naphthoquinone (Uwai et al. 2005).

The presence of dissolved organic matter (DOM) may attenuate light and slow the rate of both direct and indirect photochemical reactions through inner filtering (Lam et al. 2004). Nitrates in solution have been found to enhance the rate of phototransformation through the formation of $OH^{\bullet}$ radicals (Andreozzi et al. 2003; Crosby 1998; Schwarzenbach et al. 2003):

$$NO_3^{-} \rightarrow NO_3^{-\bullet} \rightarrow NO_2^{\bullet} + O^{\bullet-} + (H_2O) \rightarrow NO_2^{\bullet} + HO^{-} + HO^{\bullet} \qquad (2.3.17)$$

Humic acids act in two opposite effects on the photodegradation of organic molecules in water. Inner filtering is a result of the ability of humic acids to absorb UV radiation across a wide band of wavelengths. This reduces the amount of energy available to organic molecules in the solution and decreases the rate of photodegradation, as is seen for carbamazepine and diclofenac (Andreozzi et al. 2003). However, once molecules of humic acids receive UV radiation, they are promoted to a transient excited state (triplet state) where they can react with oxygen in the solution to form reactive species, such as singlet oxygen or react directly with other organic species thereby causing their phototransformation. This effect is known to cause an enhancement in the rate of phototransformation of clofibric acid (Andreozzi et al. 2003). The phototransformation rate of organic molecules will therefore depend on the balance found between these two opposite contributions caused by the presence of humic acids.

2.3.2.2 Photocatalytic Degradation

Photocatalytic degradation is used to transform, deactivate and minimize environmentally persistent compounds and affect aromatic and olefinic moieties more than aliphatic ones. Doll et al. (2004) have described it as a "promising tool for water treatment" and an "elegant and economically attractive water treatment step". Carbamazepine and clofibric acid have been eliminated through photocatalysis with TiO_2 at wavelengths less than 413 nm. Degradation is enhanced by the presence of natural organic matter (Doll et al. 2004). Natural organic matter (NOM) consists of high molecular weight water-soluble compounds (several 100 g/mol) that are ubiquitous in aquatic systems. It is also called dissolved organic carbon (DOC) in natural waters. They are responsible for many interactions during both water treatment and natural processes. Chlorination of drinking water containing NOM can lead to the formation of disinfectant by-products and can be bleached during photocatalysis (Doll et al. 2004). Natural organic matter is also important for sunlight-induced photochemical processes in surface water (Doll et al. 2004). Thermal degradation should also be considered for some compounds. Carbamazepine can be thermally degraded in a heated desolvation chamber (Lam et al. 2004). However, less degradation is seen with lower desolvation temperature and increased desolvation flow rates (Lam et al. 2004). Thermal degradation information could not be found for the other target compounds, but has the potential to be a problem.

2.3.2.3 Biotransformation

Biotransformation describes the conversion of organic and inorganic compounds to other substances within the body. Primary (Phase I) metabolism alters basic chemical structure, such as oxidation or reduction while secondary (Phase II) metabolism involves conjugation, or modification of reactive functional groups. Examples of Phase I metabolism include conversion of toluene to benzyl alcohol, benzaldehyde and benzoic acid, while examples of Phase II metabolism include conversion of benzoic acid to benzoglycine. Biotransformation is the main mechanism by which the terrestrial environment removes pollutants (Crosby 1998). Microorganisms are a major force in the transformation of chemicals and most degrade aerobically, that is, through the use of oxygen. Anaerobes use sulfur, sulfate, nitrate or bicarbonate during degradation. Many pharmaceuticals are resistant to biotransformation and may not be effectively removed during wastewater treatment (Drillia et al. 2005). Phase II metabolites in sewage effluents may be re-released as the biologically active parent compound if the conjugates are exposed to microbial activities. Schwaiger et al. (2004) state the glucoroconjugated and sulphate metabolites of diclofenac may be transformed back to the active drug when exposed to microbial activity. However, diclofenac has shown no degradation in sludge under aerobic conditions, and lake water studies suggested minimal chemical and biological degradation (Buser et al. 1998c). Caffeine was found to degrade slowly in sewage to a variety of xanthines while biotransformation is important for the degradation of ibuprofen in the environment (Buser et al. 1999; Gardinali et al. 2002; Tixier et al. 2003). In humans, caffeine was extensively metabolized, with only 3% being excreted unchanged in urine (Gardinali et al. 2002). Sixty percent of naproxen is eliminated unmetabolized and has been found in the environment (Kosjek et al. 2005). Fluoxetine is extensively metabolized in the liver by N-demethylation to norfluoxetine (Vlase et al. 2005). Shen et al. (2002) found stereoselective preferential degradation of R-fluoxetine over that of S-fluoxetine. Enantiospecific degradation has also been seen for ibuprofen. It is enantioselectively metabolized to the *S*-(+)-enantiomer in the human body, with 95% excess (Buser et al. 1999). Although the *S*-(+)-enantiomer is excreted from humans in greater concentrations than *R*-(-)-enantiomer, the *S*-(+)-enantiomer degrades faster in surface water and the sewage system (Buser et al. 1999). Buser et al. (1999)

speculate that there may be an enantiomerization process occurring in Lake Greifensee in which *S*-(+)-ibuprofen is converted into the *R*-(-)-enantiomer, based on earlier studies that found such a process for the structurally similar herbicides mecoprop and dichloroprop (Buser et al. 1998a; Buser et al. 1999).

2.3.2.4 Abiotic Transformations

Abiotic transformations involve purely chemical reactions, such as oxidation, reduction, hydrolysis and ozonation. Most environmental reactions follow pseudo-first-order kinetics.

Oxidation involves the loss of electrons but also refers to the introduction of an oxygen atom into a molecule. Dissolved oxygen from the atmosphere is able to enter into free radical reactions with compounds dissolved in the water. Hydroxyl radicals are very common oxidants in the aquatic environment and commonly formed through the photolysis of H_2O_2 and NO_3^-:

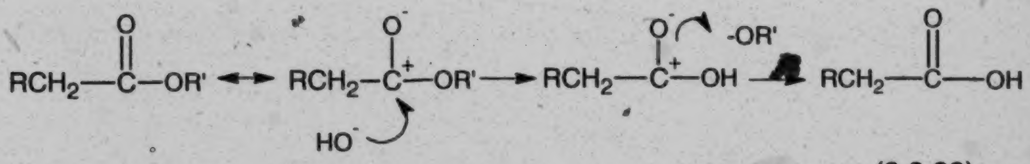
$$H_2O_2 \rightarrow 2HO\bullet \qquad \lambda<300\text{nm} \qquad (2.3.20)$$

$$NO_3^- \rightarrow NO_2^- + HO\bullet \quad \text{acidic solution, } \lambda<350\text{nm} \qquad (2.3.21)$$

These reactions cause indirect photodegradation, in which non-substrate molecules absorb the UV radiation to generate the HO• radicals. The hydroxyl radicals will react rapidly and unselectively with most organic compounds and are promising processes for the removal of pharmaceuticals in drinking water (Huber et al, 2003; Kwan et al. 2004). Oxidation should be a major pathway for the degradation of the target pharmaceuticals due to the reactivity of the hydroxyl radicals and the potential for indirect photodegradation. Ozonation and filtration through granular activated carbon has been found to be effective in removing some pharmaceuticals during drinking water treatment (Doll et al. 2004).

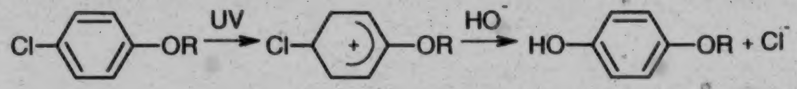
Reduction is the gain of electrons but also refers to hydrogenation, or the introduction of hydrogen into a molecule. Reduction generally takes place in anaerobically in soil pore water through water saturation and microbial metabolism (Crosby 1998). Reduction is not expected to be an important process in the fate of the target compounds.

Hydrolysis reactions are able to replace an alkoxide ion with a hydroxyl group. A negative hydroxide ion can attack the polarized carbonyl of an ester in an S_N2 reaction that displaces the alkoxide ion:



(2.3.22)

Hydrolysis is not expected to be a major degradation pathway for the target pharmaceuticals, although chlorine-containing molecules in a light-activated state can undergo alkaline hydrolysis (Crosby 1998).



(2.3.23)

2.3.3 Environmental Occurrence

2.3.3.1 Wastewater Treatment Plants

Wastewater treatment plants have been identified as a major point source for environmental discharge and surface water contamination by pharmaceuticals because of non-quantitative removal of pharmaceuticals during the treatment process (Doll et al. 2004; Ferrari et al. 2004). Water treatment processes should be developed and judged based on their potential for eliminating pharmaceuticals (Doll et al. 2004, Comoretto et al. 2005). During periods of low flow rate, such as summer, or in areas containing a high density of WWTP, effluents may account for a larger proportion of river water flow leading to increased pharmaceutical concentrations (Ashton et al. 2004; Comoretto et al. 2005; Wiegel et al. 2004). Use, pharmacokinetics, physicochemical properties of the drugs and the water treatment processes will all affect the amount of pharmaceuticals introduced by a municipal effluent discharge (Brooks et al. 2005). The deterioration of river water quality in the Mediterranean has been attributed to the presence of organic pesticides and pharmaceuticals (Andreozzi et al. 2003; Comoretto et al. 2005). Canadian WWTP have shown poor removal rates for pharmaceuticals, and it is believed water treatment efficiencies are reduced by the cold weather found during the winter months (Metcalfe et al. 2003a). The following sections outline the environmental occurrence of each pharmaceutical individually.

2.3.3.2 Ibuprofen

Ibuprofen has been found in the environment in many countries, including Switzerland, Spain, Brazil, Greece, the United States, the United Kingdom and

Canada. Ibuprofen is efficiently eliminated in WWTP (75-99.9%) (Buser et al. 1999; Tixier et al. 2003). A study by Buser et al. (1999) found ibuprofen levels of 1-3.3μg/L in WWTP influents and 2-81ng/l in WWTP effluents. The major metabolites of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen, were found in both influent and effluent samples (Buser et al. 1999). A Brazilian study found ibuprofen to be more efficiently removed using an activated sludge treatment (75%), as compared to biological filtration (22%) (Ashton et al. 2004). It is efficiently removed during the wastewater treatment process, although some residues have been detected in various rivers, lakes and streams (Weigel et al. 2004a). In German rivers and streams over the period of 1996 to 1998, ibuprofen was seen at an average of 0.07 μg/L, with a maximum concentration of 0.53 μg/L (Ternes 2001). Metcalfe et al. (2003) indicate ibuprofen is removed with >90% efficiency in sewage treatment plants (STPs) where sewage was retained for 12 hours or more. However, in a 2002 study of the Lower Great Lakes region of Canada, Metcalfe et al. (2003) found concentrations of up to 0.027 μg/L in surface waters near WWTP. In 1998-1999, Metcalfe found median values of 38.7 μg/L in influent samples and 4.0 μg/L in effluent samples in fourteen Canadian WWTPs (Metcalfe et al. 2003a). Ashton et al. (2004) reported ibuprofen concentrations from England and Wales, with mean values of 4 μg/L in WWTP effluents, a maximum of 27 μg/L occurring at the Great Billing WWTP and surface water concentrations of up to 5 μg/L. A Norwegian study detected ibuprofen and its metabolites in sewage effluents that included hospital effluent with summed concentrations in the range 0.1-29 μg/L as well as in seawater with values up to 7.7 ng/L (Weigel et al. 2004a). In 1998, Wiegel et al. (2004) found ibuprofen in the range of <20 ng/L to 70 ng/L in the Elbe River, one of the major rivers in central Europe, and its tributaries (Weigel et al. 2004a). In 2000, ibuprofen concentrations ranged from <2 to 146 ng/L in the Elbe River (Weigel et al. 2004a). Buser et al. (1999) detected ibuprofen concentrations of 1-3.3 µg/L in the influents of WWTP at Gossau, Pfäffikon and Uster (Buser et al. 1999). The corresponding effluent concentrations were much lower, at 2-81 ng/L (Buser et al. 1999). The influent ibuprofen showed a predominance of *S*-ibuprofen, with an enantiomer ratio (ER = S/R) of 5.5 – 8 (Buser et al. 1999). The effluents showed a much lower ER of 0.9 – 2, indicating S-ibuprofen is degraded faster than *R*-ibuprofen during the treatment process (Buser et al. 1999). Lake Greifensee had ibuprofen levels of 2 – 8 ng/L, with ER of 2.1 (Buser et al. 1999). One location investigated upstream from the WWTP had a concentration of <0.2 ng/L, confirming the WWTP was the source of ibuprofen contamination (Buser et al. 1999). Lakes Zurichsee, Baldeggersee and Pfäffikersee showed ibuprofen concentrations of 3.3-4.0 ng/L, 1.5-3.2 ng/L and 4.0 ng/L, respectively, with ERs of up to 2.0 but ibuprofen was not seen in Lake Sempachersee or in the North Sea (Buser et al. 1999). Municipal German WWTP effluents over the period from 1996 to 1998 had average ibuprofen concentrations of 0.37 µg/L, with a maximum concentration of 3.4 µg/L (Ternes 2001).

2.3.3.3 Clofibric Acid

Clofibric acid is commonly found in the environment, having been found in the North Sea, rivers, ground water and even drinking water at concentrations of up to 270 ng/L (Hernando et al. 2004; Weigel et al. 2004a). A study by Buser et al. (1998) found clofibric acid at 7.8 ng/L in the North Sea and at 1-9 ng/L in Lakes Sempachersee, Zurichsee and Greifensee in Switzerland (Buser et al. 1998b). To be found in drinking water, a drug must survive the wastewater treatment process as well as drinking water treatment steps, including filtration and disinfection. Clofibric acid does not degrade well during wastewater treatment, with only 34% to 50% removed from a conventional WWTP (Buser et al. 1999; Hernando et al. 2004). A 2002 Canadian study found clofibric acid concentrations of 0.044 µg/L in Peterborough, ON and 0.002 µg/L in Burlington, ON WWTP effluents as well as 0.001 µg/L in Hamilton Harbour and 0.003 µg/L in Little River surface waters adjacent to discharges of effluents from WWTP (Metcalfe et al. 2003b). In three Switzerland WWTP effluents, concentrations reached 0.06 µg/L (Tixier et al. 2003). Buser et al. (1999) found clofibric acid concentrations of 6-105 ng/L in WWTP at Gossau, Pfäffikon and Uster in Germany. Municipal German WWTP effluents over the period from 1996 to 1998 had average clofibric acid concentrations of 0.36 µg/L, with a maximum concentration of 1.6 µg/L and a limit of quantification of 0.050 µg/L (Ternes 2001). Clofibric acid concentrations of 1-2 ng/L have been detected in the North Sea (Buser et al. 1998b). It has been found over the whole German Bight with a uniform concentration of 0.5-2.4 ng/L and has been detected in surface water, groundwater and drinking water in areas that had been used as sewage farms in and around Berlin (Buser et al. 1998b). In German rivers and streams over the period of 1996 to 1998, clofibric acid was seen at an average of 0.066 µg/L, with a maximum concentration of 0.55 µg/L (Ternes 2001). Due to a replacement of clofibric acid by newer drugs like gemfibrozil, fenofibrate and benzafibrate, it has not been detected in France (Comoretto et al. 2005).

2.3.3.4 Caffeine

Caffeine has been found even in remote locations that have very little human settlement such as Vengsoyfjorden and Kaldfjorden in Norway (Weigel et al. 2004a). Despite the low percentage of unmetabolized caffeine excreted, and the assumption that some of the remainder will be removed during waste treatment, caffeine is found in many surface waters. Metcalfe et al. (2003) found caffeine in concentrations as low as 0.014 µg/L in the Otonabee River and as high as 0.046 µg/L in the Detroit River during a 2002 survey of the Lower Great Lakes region of Canada. The same survey found Ontario WWTP concentrations of 0.022 µg/L for Peterborough and Burlington, 0.028 µg/L for Little River and 0.677 µg/L for West Windsor (Metcalfe et al. 2003b). Swiss wastewater treatment plant effluents were found to have caffeine concentrations of 0.03-9.5 µg/L, with an estimated removal rate of >80% (Buerge et al. 2003), associated with microbial degradation. The same study found concentrations in Swiss lakes and rivers of 6 ng/L to 250 ng/L with remote mountain lakes having <2 ng/L (Buerge et al. 2003). Caffeine was detected in Mediterranean Sea surface waters at 4-5 ng/L (Buerge et al. 2003). In Iowa in 2001, Kolpin et al. (2004) found caffeine values of 0.078 µg/L, 0.036 µg/L and 1.39 µg/L for high-flow, normal-flow and low-flow sampling periods, respectively (Kolpin et al. 2004). In Tromsø, Norway, caffeine was found in a WWTP influent at 54.7 µg/L and in the effluent at

47.7 µg/L (Weigel et al. 2004a). The maximum concentration found in that study was 293 µg/L in the Breivika Hospital sewer (Weigel et al. 2004a). Caffeine was found in Deal Lake, New Jersey, USA at up to 44 µg/L during wet weather and up to 0.267 µg/L in dry weather (Sankararamakrishnan et al. 2005).

2.3.3.5 Fluoxetine

Fluoxetine was not detected in any influents or effluents in a Norwegian study that did detect ibuprofen, diclofenac, clofibric acid, caffeine, carbamazepine and other drugs (Weigel et al. 2004a). Similarly, the Kolpin study of 2001 found no trace of fluoxetine in any of the 76 water samples sampled in Iowa (Kolpin et al. 2004). Fluoxetine was found in Ontario WWTPs in 2002 at concentrations of 0.050 µg/L, 0.038 µg/L and 0.099 µg/L for the Peterborough, Burlington and Little River WWTPs, respectively (Metcalfe et al. 2003b). The same study found 0.013 µg/L and 0.046 µg/L in Hamilton Harbour and Little River surface waters adjacent to WWTP effluents (Metcalfe et al. 2003a). Despite the long half-life of norfluoxetine, it was not found in any of the waters studied. No chiral data could be found for this chiral metabolite.

2.3.3.6 Gemfibrozil

Gemfibrozil has been found in some Canadian sewage treatment plants that have been analyzed. The removal of gemfibrozil and other lipid regulating drugs from WWTPs is between 34 and 50%, and traces have been found in sewage effluents, groundwater and surface waters (Hernando et al. 2004). Hernando et al. (2004) found around 30% recovery in spiked influent and effluent water samples, with a limit of detection of 0.090 µg/L. A similar study by Miao et al. (2002) found a 78% recovery from spiked WWTP effluent with a limit of detection of 5 ng/L. A German study found an 89% recovery from spiked surface water, with a 17 ng/L limit of detection (Sacher et al. 2001). Municipal German WWTP effluents over the period from 1996 to 1998 had average gemfibrozil concentrations of 0.40 µg/L, with a maximum concentration of 1.5 µg/L and a limit of quantification of 0.050 µg/L (Ternes 2001). Cahill et al. (2004) detected a median gemfibrozil concentration of less than 0.20 µg/L. In the Great Lakes region of Canada, gemfibrozil was found in the Detroit River and Hamilton Harbour surface waters with median values of 0.066 µg/L and 0.012 µg/L, respectively, during a 2000 study (Metcalfe et al. 2003b). In German rivers and streams over the period of 1996 to 1998, gemfibrozil was seen at an average of 0.052 µg/L, with a maximum concentration of 0.51 µg/L and a limit of quantification of 0.010 µg/L (Ternes 2001).In a 2002 study of sewage treatment plants in Ontario, Canada, Metcalfe et al. (2003) found 1.493 µg/L, 0.005 µg/L, 0.012 µg/L and 0.043 µg/L in the Peterborough, Burlington, Little River and West Windsor WWTP effluents, respectively. This same study examined surface waters adjacent to effluents discharged by WWTPs and found 0.038 µg/L, 0.034 µg/L and 0.002 µg/L in Hamilton Harbour, Little River and Detroit River surface waters. Gemfibrozil was not detected in a 2001 study of Iowa waters (Kolpin et al. 2004).

2.3.3.7 Diclofenac

Diclofenac has been found in Germany in sewage effluents and the surface waters near sewage treatment plants at levels (Metcalfe et al. 2003a).

Ashton et al. (2004) reported diclofenac concentrations of 424 ng/L and <20 ng/L for final effluent and surface waters in the United Kingdom, respectively, after primary sedimentation, aeration and phosphate removal. Two very effective methods for removing diclofenac are granular activated carbon and ozonation (Ternes et al. 2002; Vogna et al. 2004). Diclofenac is eliminated in surface waters by phototransformation as well as in the epilimnion, the layer of water above the thermocline, in which it had an eight-day half-life time (Tixier et al. 2003). The authors did point out that phototransformation processes are strongly time and site specific and may vary by orders of magnitude (Tixier et al. 2003). Diclofenac is considered non-persistent because it had a half-life of <1 day in the aquatic environment (Ashton et al. 2004; Poiger et al. 2001). In a Swiss study, concentrations of diclofenac reached 0.99 µg/L in wastewater treatment plant effluents while Lake Greifensee in Switzerland was found to have an average diclofenac removal rate of 0.088±0.012 day^{-1} (Tixier et al. 2003). Lake Greifensee in Switzerland had much higher concentrations in a major tributary (up to 370 ng/L) than in the outflow (up to 12 ng/L) (Comoretto et al. 2005).

A European study found maximum diclofenac effluent concentrations of 0.29 µg/L in France while in Germany, it found 2 µg/L in effluents and 1.03 µg/L in surface waters (Ferrari et al. 2004). A study of the Elbe River and its tributaries in central Europe in 1998 found concentrations in the range 10 to 59 ng/L (Weigel et al. 2004a). A 2000 study of the same river found a range of <1 to 69 ng/L (Weigel et al. 2004a). In German rivers and streams over the period of 1996 to 1998, diclofenac was seen at an average of 0.15 µg/L, with a maximum concentration of 1.20 µg/L and a limit of quantification of 0.010 µg/L (Ternes 2001). Diclofenac was found with a median concentration of 0.6 µg/L in WWTP effluent and 0.154 µg/L downstream during a United Kingdom study (Ashton et al. 2004). A Canadian study in 1998-1999 found a maximum concentration of 1.3 µg/L in WWTP influents but no drug in the effluents (Metcalfe et al. 2003a). Municipal German WWTP effluents over the period from 1996 to 1998 had average diclofenac concentrations of 0.81 µg/L, with a maximum concentration of 2.1 µg/L and a limit of quantification of 0.050 µg/L (Ternes 2001). A 2000 survey of the Great Lakes region of Canada found maximum concentrations of 0.042 µg/L and 0.194 µg/L in Detroit River and Hamilton Harbour surface waters, respectively (Metcalfe et al. 2003b). The same study found Ontario WWTP effluent values of 0.359 µg/L in Peterborough, 0.005 µg/L in Burlington, 0.088 µg/L in Little River and 0.063 µg/L in West Windsor (Metcalfe et al. 2003b). The surface waters adjacent to these WWTPs saw 0.018 µg/L in Hamilton Harbour and 0.050 µg/L in Little River but no drug was detected in Otonabee River or Detroit River (Metcalfe et al. 2003b). Sacher et al. (2001) found a maximum concentration of 590 ng/L in Baden-Württemberg, Germany ground waters. Diclofenac was found at levels of 310-930 ng/L from WWTP effluents at Gossau, Pfäffikon and Uster (Buser et al. 1998c). In a later study of the same area, diclofenac levels reached 310-1920 ng/L in WWTP at Gossau, Pfäffikon and Uster and has also been detected in rivers and lakes in Switzerland (Buser et al. 1998c, 1999). Diclofenac is degraded through photodegradation in the lake, not in the WWTP (Buser et al. 1999; Poiger et al. 2001).

2.3.3.8 Naproxen

It has been suggested that naproxen is eliminated very efficiently (>90%) when the sewage is retained in the WWTP for at least 12 hours (Metcalfe et al. 2003b). However, a Swiss study found levels of naproxen reaching 2.6 µg/L in WWTP effluents (Tixier et al. 2003). This same study found a naproxen removal rate of 0.05 day^{-1} in Lake Greifensee, with a half-life of 14 days (Tixier et al. 2003). Metcalfe et al. (2003) found an average of 40.7 µg/L and a maximum of 611 µg/L of naproxen in the influents of 14 Canadian WWTP in 1998, considerably higher than the corresponding effluent values of 12.5 µg/L (average) and 33.9 µg/L (maximum). The study did indicate the concentrations seen were higher than those previously reported in German WWTP effluents (Metcalfe et al. 2003a). Municipal German WWTP effluents over the period from 1996 to 1998 had average naproxen concentrations of 0.30 µg/L, with a maximum concentration of 0.52 µg/L and a limit of quantification of 0.050 µg/L (Ternes 2001). In 2000, naproxen was found in the surface water of Detroit River and Hamilton Harbour in concentrations up to 0.551 µg/L and 0.139 µg/L, respectively (Metcalfe et al. 2003b). In German rivers and streams over the period of 1996 to 1998, naproxen was seen at an average of 0.07 µg/L, with a maximum concentration of 0.39 µg/L and a limit of quantification of 0.010 µg/L (Ternes 2001). Ontario WWTP effluent concentrations were found to be 0.524 µg/L, 0.041 µg/L, 0.021 µg/L and 0.168 µg/L for Peterborough, Burlington, Little River and West Windsor, respectively (Metcalfe et al. 2003b). In the surface waters adjacent to these WWTPs, naproxen was found in Hamilton Harbour and

Little River at concentrations of 0.039 µg/L and 0.073 µg/L (Metcalfe et al. 2003b). No information could be found regarding chiral inversion of naproxen.

2.3.3.9 Indomethacin

In a study of Ontario WWTP effluents, indomethacin was found with concentrations of 0.378 µg/L, 0.010 µg/L and 0.021 µg/L in Peterborough, Burlington, and Little River, respectively (Metcalfe et al. 2003b). In the surface waters adjacent to these WWTPs, indomethacin was only found in Hamilton Harbour and Little River at levels of 0.005 µg/L and 0.018 µg/L, respectively (Metcalfe et al. 2003b). Municipal German WWTP effluents over the period from 1996 to 1998 had average indomethacin concentrations of 0.27 µg/L, with a maximum concentration of 0.60 µg/L and a limit of quantification of 0.050 µg/L (Ternes 2001). In German rivers and streams over the period of 1996 to 1998, indomethacin was seen at an average of 0.04 µg/L, with a maximum concentration of 0.20 µg/L and a limit of quantification of 0.010 µg/L (Ternes 2001).

2.3.3.10 Fenoprofen

In a 1998-1999 study of 14 Canadian sewage treatment plants, fenoprofen was found with median and maximum concentrations of 1.8 µg/L and 9.7 µg/L in WWTP influents although it was not detected in the effluents (Metcalfe et al. 2003a). Metcalfe et al. (2003) studied the Lower Great Lakes region of Canada in 2000 and found surface water concentrations up to 0.064 µg/L in Hamilton Harbour and 0.059 µg/L in other sites. A study of Ontario WWTP in 2002 found 0.405 µg/L, 0.062 µg/L and 0.075 µg/L in Peterborough, Burlington and Little

River, respectively (Metcalfe et al. 2003b). In the surface waters adjacent to these WWTPs, fenoprofen was found only in Hamilton Harbour and Little River at concentrations of 0.142 µg/L and 0.132 µg/L (Metcalfe et al. 2003b). No information could be found regarding the chiral nature of fenoprofen in the environment.

2.3.3.11 Ketoprofen

In the effluents of a Swiss wastewater treatment plant (WWTP), ketoprofen was found in concentrations up to 0.18 µg/L but was not found in the water column of Lake Greifensee (Tixier et al. 2003). In a 2000 study of surface waters in the Great Lakes region of Canada, ketoprofen was found at concentrations up to 0.017 µg/L, 0.047 µg/L and 0.050 µg/L in Detroit River, Hamilton Harbour and other sites, respectively (Metcalfe et al. 2003b). In a 2002 study of WWTP effluents in the same region, ketoprofen was found only in Burlington, ON at a concentration of 0.013 µg/L (Metcalfe et al. 2003b). It was not found in the corresponding study of surface waters adjacent to these WWTPs (Metcalfe et al. 2003b). In 1998-1999, Metcalfe et al. (2003) found ketoprofen in 14 Canadian WWTP influent samples at 5.7 µg/L but was not detected in the effluent samples (Metcalfe et al. 2003a). Municipal German WWTP effluents over the period from 1996 to 1998 had average ketoprofen concentrations of 0.20 µg/L, with a maximum concentration of 0.38 µg/L and a limit of quantification of 0.050 µg/L (Ternes 2001). In German rivers and streams over the period of 1996 to 1998, ketoprofen was seen at a maximum concentration of 0.12 µg/L and a limit of quantification of 0.010 µg/L (Ternes 2001). No information could be found regarding the chiral nature of ketoprofen in the environment.

2.3.3.12 Metoprolol

In a German WWTP, metoprolol was removed at 83% (Ternes 1998). The maximum effluent concentration of metoprolol in this WWTP was 0.59 µg/L (Ternes 1998). Huggett et al. (2003) found an average value of 0.074 µg/L and a maximum value of 1.20 µg/L in WWTP effluents in Mississippi, New York and Texas. Stolker et al. (2004) analyzed drinking water, groundwater and surface water samples in the Netherlands and found two samples with concentrations <25 ng/L and five samples with concentrations between 25-100 ng/L. Metoprolol was found in Kallby, Sweden WWTP influents and effluents at 0.16 and 0.19 µg/L, respectively, with an 83% removal efficiency (Bendz et al. 2005). The same study reported concentrations of 0.16 µg/L in the Hoje River (Bendz et al. 2005). Municipal German WWTP effluents over the period from 1996 to 1998 had average metoprolol concentrations of 0.73 µg/L, with a maximum concentration of 2.2 µg/L and a limit of quantification of 0.025 µg/L (Ternes 2001). In German rivers and streams over the period of 1996 to 1998, metoprolol was seen at an average of 0.045 µg/L, with a maximum concentration of 2.2 µg/L and a limit of quantification of 0.010 µg/L (Ternes 2001). Andreozzi et al. (2003) studied WWTP in France, Greece, Italy and Sweden and found median and maximum concentrations of 0.08 µg/L and 0.39 µg/L, respectively. No information could be found regarding the chiral nature of metoprolol in the environment.

2.3.3.13 Atenolol

Castiglioni et al. (2005) analyzed several WWTP in Italy and found atenolol levels of 254 ng/L, 27 ng/L, 260 ng/L, 70 ng/L, 955 ng/L, 1168 ng/L, 554 ng/L and 466 ng/L in Cagliari, Cosenza, Palermo, Latina, Naples, Cuneo, Varese Lago and Varese Olona, respectively. A different study of Italian WWTP showed an atenolol concentration of 466 ng/L (Zuccato et al. 2005). Atenolol was found in Kallby, Sweden WWTP influents and effluents at 0.03 and 0.16 µg/L, respectively (Bendz et al. 2005). The same study reported concentrations of 0.03 µg/L in the Hoje River (Bendz et al. 2005). A study of the Po and Lambro rivers in Italy found atenolol concentrations ranging from 3.44 ng/L to 241 ng/L (Calamari et al. 2003). A Zuccato et al. (2005) study found 241 ng/L in the Lambro River and 41.7 ng/L and 17.2 ng/L in the Po River. No information could be found regarding the chiral nature of atenolol in the environment.

2.3.3.14 Propranolol

In a German WWTP, propranolol was removed at 96% (Ternes 1998). The maximum effluent concentration of propranolol in this WWTP was 2.2 µg/L (Ternes 1998). Municipal German WWTP effluents over the period from 1996 to 1998 had average propranolol concentrations of 0.17 µg/L, with a maximum concentration of 0.29 µg/L and a limit of quantification of 0.025 µg/L (Ternes 2001). In German rivers and streams over the period of 1996 to 1998, propranolol was seen at an average of 0.012 µg/L, with a maximum concentration of 0.59 µg/L and a limit of quantification of 0.010 µg/L (Ternes 2001). Huggett et al. (2003) found an average value of 0.117 µg/L and a maximum value of 1.90 µg/L in WWTP effluents in Mississippi, New York and Texas. Propranolol was found in Kallby, Sweden WWTP influents and effluents at 0.05 and 0.03 µg/L, respectively, with a 96% removal efficiency (Bendz et al. 2005). The same study reported concentrations of 0.05 µg/L in the Hoje River (Bendz et al. 2005). Andreozzi et al. (2003) studied WWTP in France, Greece, Italy and Sweden and found median and maximum concentrations of 0.01µg/L and 0.09 µg/L, respectively. In United Kingdom WWTP effluent samples, propranolol was found at 75 ng/L, and 29 ng/L in the corresponding receiving waters (Ashton et al. 2004). Upstream of the WWTP in this study, propranolol was found at <10 ng/L. (Ashton et al. 2004). The Howdon WWTP along the Tyne estuary, UK had average propranolol concentrations of 83 ng/L, 20 ng/L and 17 ng/L in raw influent, pre-UV water and final effluent, respectively (Roberts et al. 2005). Fono et al. (2005) found U.S. WWTP influent concentrations ranging from 13 ng/L to 250 ng/L with racemic EFs ranging from 0.49 to 0.54. After biological treatment, propranolol concentrations ranged from 3 ng/L to 160 ng/L with nonracemic EFs ranging from 0.31 to 0.44 in WWTP effluents (Fono et al. 2005). Fono et al. (2005) found propranolol concentrations in U.S. surface waters ranging from <0.1 ng/L to 32 ng/L with EFs ranging from 0.21 to 0.53.

Chapter 2.4 Analytical Measurements

2.4.1 Extraction of Analytes

The fact that most pharmaceuticals are polar and designed to be bioactive at low concentrations presents some analytical challenges. The most difficult compounds to isolate and detect include polar, amphoteric and low molecular weight compounds (Cahill et al. 2004; Zuehlke et al. 2004). The analytical techniques used to measure these drugs in environmental samples require sensitivity and selectivity, such as LC-MS/MS and to a lesser extent, GC-MS/MS (Hernando et al. 2004; Loffler et al. 2003; Miao et al. 2002). Pharmaceuticals have been extracted from gel formulations using sonication and centrifugation in acetonitrile or methanol and from tablet formulations using dissolution in ultrapure water, methanol, NH_3 or NaOH followed by filtration and dilution (Damiani et al. 2002; Doll et al. 2004; Dvorak et al. 2004; Novakova et al. 2005). Pharmaceuticals are also commonly extracted from urine, blood and environmental samples (Arcelloni et al. 2001; Aresta et al. 2005).

For best results, urine, blood and environmental samples must be treated to remove matrix components and concentrate the analytes (Ahrer et al. 2001; Flores et al. 2005; Glowka et al. 2005). Methods such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are used to clean up matrices and quantitatively extract analytes from the samples (Ahrer et al. 2001; Gardinali et al. 2002; Glowka et al. 2005). Derivitization has been used to reduce the polarity of compounds that otherwise cannot be extracted from water samples. Once derivatized, the drugs are extractable using solid phase extraction (SPE)

(Zuehlke et al. 2004). Because of its ability to concentrate pharmaceuticals from large volumes of water, SPE is the most highly used extraction method for the target pharmaceuticals. One advantage includes the minimal use of organic solvents, which is a major downfall in LLE. A general SPE method for pharmaceuticals is as follows. The SPE cartridge is primed and the aqueous sample is applied to the cartridges, then allowed to pass through the sorbent until the cartridge is dry. Excess water may be removed using centrifugation or the cartridges may be allowed to dry under vacuum. Organic solvent is added to the dried cartridges to elute the analytes. The eluent may be passed through a drying column, then filtered through a syringe filter to remove any particulate matter. The resulting solution is concentrated, internal standard is added and the sample can now be analyzed.

Many types of SPE adsorbents are available, including Empore C_{18}, LiChrolut RP 18, LiChrolut EN, Bio-Beads, Isolut RP-C_{18}, Bondesil ODS and Oasis HLB (Ahrer et al. 2001; Alvarez et al. 2004; Andreozzi et al. 2003; Bendz et al. 2005; Boyd et al. 2004; Brooks et al. 2003; Buerge et al. 2003; Buser et al. 1998c, 1999; Calamari et al. 2003; Ferrari et al. 2004). These have all been used for the target pharmaceuticals, including fluoxetine, caffeine, carbamazepine, clofibric acid, diclofenac, naproxen and ibuprofen (Ahrer et al. 2001; Andreozzi et al. 2003; Brooks et al. 2003; Buerge et al. 2003; Ferrari et al. 2004). Although Ahrer et al. (2001) found Bondesil ODS gave the best average recovery (60%) of the five adsorbents tested, Oasis HLB is generally the adsorbent of choice for acidic pharmaceuticals, with recoveries often >90%

(Ahrer et al. 2001; Alvarez et al. 2004; Cahill et al. 2004; Kloepfer et al. 2005; Lam et al. 2004; Lin et al. 2005; Miao et al. 2003; Ollers et al. 2001; Quitana et al. 2004; Sabbioni et al. 2004; Shen et al. 2005; Tixier et al. 2003; Weigel et al. 2004a; Weigel et al. 2004b). Neutral and acidic pharmaceuticals can be concentrated using adsorbents that allow van der Waals and hydrogen bonding interactions (Ollers et al. 2001). The Oasis HLB sorbent (polystyrene-divinylbenzene-N-vinylpyrrolidine terpolymer) has both hydrophilic and lipophilic characteristics, making it a useful sorbent for a wide range of compounds. The lipophilic polystyrene-divinylbenzene portion provides the requisite van der Waals and hydrogen bonding interactions needed to sorb the target analytes, while the hydrophilic N-vinylpyrrolidine monomer is responsible for the easy-wetting properties of the adsorbent (Ollers et al. 2001). Care must normally be taken to prevent SPE cartridges from running dry because specific sequences involving both organic and aqueous solvents are needed to prime the cartridges before use. The manufacturer recommends methanol followed by water to prime the Oasis HLB cartridges. Priming is necessary to wet the cartridge so analyte can sorb to the available sites and if it dries out, the sites may not be available for sorption. Oasis HLB cartridges are generally resistant to this problem however, because the hydrophobic sorbent is water wettable and a study by Ollers et al. (2001) found running the cartridge dry had no negative effect on analyte recovery. Acidic targets are normally acidified before extraction to protonate the carboxylic acid groups, thereby removing the negative charge that may interfere with extraction from the aqueous sample. However, Cahill et al. (2004) found

Oasis HLB cartridges gave the best overall extraction recovery for a variety of pharmaceuticals, including fluoxetine, ibuprofen and gemfibrozil, when no pH adjustments were used. The typical elution solvent used for the target analytes is methanol, despite the Ollers et al. (2001) study that found absolute recoveries much less than 100% for ketoprofen, diclofenac and carbamazepine when using methanol. A 50:50 ethyl acetate/acetone mixture was optimal for extraction, followed by derivitization and GC-MS analysis (Ollers et al. 2001). However, these relatively harsh organic solvents are not compatible with reverse phase liquid chromatography, because the columns and pump seals are susceptible to degradation by these solvents.

In spite of its wide use, there are some problems associated with SPE. Ahrer et al. (2001) states that quantitative adsorption to the stationary phase is a necessary condition of the final SPE conditions chosen. However, if the SPE recoveries are reproducible, then non-quantitative adsorption can be accounted for through the use of surrogate standards. As discussed above, acidification of samples is often used to aid in adsorption, and the specific method used for the SPE must be optimized. Certain analytes, such as diclofenac and ibuprofen, have relatively poor solubility in organic solvents that may impact the elution and redissolution of target analytes (Ahrer et al. 2001). However, the typical environmental concentrations (ng/L) are far below typical solubility limits (mg/L), so this is not expected to be an issue. In addition to adsorbing to the SPE sorbent, targets containing amine functional groups may adsorb to any glassware used and can negatively affect recoveries (Ahrer et al. 2001). The adsorption can be reduced through silanization of the glassware using dimethyldichlorosilane (Ahrer et al. 2001). However, no other studies were found that used this process. Additionally, spike and recovery experiements resulting in reasonable recoveries are being seen, even for complex sample matrices (Cahill et al. 2004; Huggett et al. 2003; Rodriguez et al. 2003). Thus, competitive sorption to glassware is not expected to be a problem. Dissolved organic carbon (DOC), such as humic acids, depends on the water sample and can make sample pre-treatment more difficult when high recoveries are desired (Ahrer et al. 2001; Antignac et al. 2005; Shen et al. 2005). Drinking water in Austria, for example, contains ~2 mg/L DOC while surface river waters in Upper Austria contains up to 20 mg/L DOC (Ahrer et al. 2001). As a comparison, Huber et al. (2003) found DOC levels of 1.2 mg/L, 1.3 mg/L, 0.8 mg/L and 3.7 mg/L for lake Zurichsee in Switzerland, River Seine in France, well water from Porrentruy, Switzerland, and a lake in Finland, respectively. Untreated municpal wastewater in Germany was found to have DOC of up to 57 mg/L (Kloepfer et al. 2005). If the water matrix contains compounds that are easily extractable, SPE may lead to an increased concentration of interferences and a magnification of matrix effects, which could be accounted for by diluting extracts before analysis (Hernando et al. 2004).

2.4.2 Measurement of analytes

2.4.2.1 Achiral Gas Chromatography

Chromatography is typically used to separate the components in extracted samples. Compounds are separated based on their affinity for the stationary and mobile phases. Gas chromatography (GC) has been used for both chiral and achiral pharmaceutical analysis, but is not effective for non-volatile compounds such as the organic acids studied in this thesis (Berzas et al. 2004; Boyd et al. 2004; Buerge et al. 2003; Buser et al. 1998b; Buser et al. 1999; Carpinteiro et al. 2004; D'Orazio et al. 2005; Ferrari et al. 2004; Huggett et al. 2003; Kosjek et al. 2005; Lin et al. 2005; Petrovic et al. 2005a; Sacher et al. 2001; Scheytt et al. 2005; Ternes et al. 2005; Tixier et al. 2003; Weigel et al. 2004a; Weigel et al. 2004b). As such, labour-intensive derivitization is required to increase the volatility and may involve preparation of a derivitization agent such as diazomethane.

Diazomethane has been used to methylate pharmaceuticals with carboxylic groups, such as clofibric acid, diclofenac, fenoprofen, gemfibrozil, ibuprofen, ketoprofen and naproxen for subsequent GC analysis. However, diazomethane is toxic, carcinogenic and explosive and should be generated before each use (Andreozzi et al. 2003; Buser et al. 1998b; Buser et al. 1998c, 1999; Lin et al. 2005). N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), tetrabutylammonium hydrogen sulfate (TBA-HSO_4) and pentafluorobenzyl bromide (PFBBr) have also been used as derivitizing agents for the target pharmaceuticals (Carpinteiro et al. 2004; Kosjek et al. 2005; Lin et al. 2005; Scheytt et al. 2005). Fluoxetine and caffeine have been analyzed using GC without derivitization because they are more volatile than the organic acids studied (Berzas et al. 2004).

2.4.2.2 Capillary Electrophoresis

Capillary electrophoresis (CE) is gaining in popularity in the analysis of phamaceuticals due to its simplicity, rapidity, high efficiency and resolution and minimal use of reagents (Ammazzalorso et al. 2005). Some work has been done on separation of pharmaceuticals such as clofibric acid, ibuprofen, naproxen and diclofenac in both chiral and achiral applications, but it is still not as commonly used as chromatographic methods (Ahrer et al. 2001; Ammazzalorso et al. 2005; D'Orazio et al. 2005; Flores et al. 2005; Glowka 2002; Gubitz et al. 2001). Since analytes must be ionized during CE, the use of CE is traditionally limited to analytes with pKa values that allow deprotonation under typical aqueous CE conditions (Ahrer et al. 2001). A limiting step in the use of CE with aqueous buffers could be the water solubility of certain pharmaceuticals, but this is being addressed through the use of nonaqueous buffers (Flores et al. 2005). Capillary electrophoresis has proven to be amenable to ESI, and work has been done using CE-MS (Ahrer et al. 2001). CE typically shows higher detection limits than LC, but this can be detector dependant. Ketoprofen enantiomers determined using CE with UV detection had a method limit of detection (LOD) of 0.1 mg/L and a limit of quantification (LOQ) in serum of 0.25 mg/L and ibuprofen enantiomers enhibited LOD of 0.05 mg/L and 0.25 mg/L in serum and urine;

respectively (Glowka 2002; Glowka et al. 2005). Fluoxetine was determined using achiral CE with a LOD of 10 µg/L (Flores et al. 2005). In comparison, typical LC detection limits are on the order of ng/L.

2.4.2.3 Achiral Liquid Chromtography

Liquid chromatography (LC) is the most commonly used chromatographic method for the analysis of thermally labile and non-volatile compounds such as the organic acid pharmaceuticals investigated in this thesis. It has proven useful for the routine analysis of pharmaceuticals because of its reproducibility, robustness and ability to determine ultra trace concentrations (part-per-trillion) of many pharmaceuticals in wastewater, surface water and ground water (Cahill et al. 2004; Carpinteiro et al. 2004; D'Orazio et al. 2005; Hernando et al. 2004). When coupled with a mass spectrometer, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is one of the most powerful analysis techniques available for quantitation and structural identification due to its sensitivity, specificity and selectivity (Hernando et al. 2004). It has also been useful for determination and quantification of drug metabolites in biological matrices (Chen et al. 2005).

Pharmaceuticals are most commonly analyzed using reverse-phase HPLC. There are many achiral columns available commercially, including Metasil Basic, Phenosphere ODS, Supelco Discovery C_{18}, Zorbax ODS, Spherisorb ODS II, Purospher Star RP-18 and Luna phenyl-hexyl (Cahill et al. 2004; Damiani et al. 2002; Dvorak et al. 2004; Hermening et al. 2000; Hernando et al. 2004; Kloepfer et al. 2005). Common organic solvents used are methanol and acetonitrile, while aqueous buffer additives include formic acid and trifluoroacetic acid for protonation of analytes during chromatography, and salts such as ammonium acetate and ammonium trifluoroacetate. These additives aid in the separation of the analytes by buffering the mobile phase and providing reproducible results. Salts can affect analyte ionization during mass spectrometry (MS). Trifluoroacetic acid (TFA), for example, causes suppression of negative mode analytes because the organic acids will not deprotonate effectively in acidic solution (Applied 2005). Triethylamine (TEA) may enhance negative mode ionization but suppress the ionization of less basic compounds in positive mode (Applied 2005). Buffering works best when the pH is within ±1 unit of the analyte pKa. Polar organic mode is also used, and consists of methanol or acetonitrile with a small percent of volatile additives such as acetic acid, TEA or TFA. A combination of acidic and basic additives may result in neutralization of analyte ions preventing their ionization and thus analysis by MS.

2.4.2.4 Chiral Liquid Chromatography

2.4.2.4.1 Indirect Chiral Separation

Given the importance of chirality in biological processes and thus in the pharmaceutical industry, chiral separation is important for chromatography. Chiral derivitization can be used indirectly to form chiral derivatives that differ in chemical and physical properties, allowing separation on achiral stationary phases (Gubitz et al. 2001). This avoids the use of expensive chiral columns at the expense of an added reaction step that may form undesired side products and may not proceed to 100% (Gubitz et al. 2001; Haginaka 2002). The optical purity of the derivitization reagent must be known or the optical purity of the target compound will not be determined precisely (Haginaka 2002). This indirect method is not suitable for enantiomers in standard solutions or pharmaceutical preparations but does work for trace analysis in biological matrices (Haginaka 2002). When a chiral target reacts with a chiral reactant, the result is two pairs of enantiomers. The two enantiomers of each new pair will overlap when using achiral stationary phases because they are enantiomeric pairs. In complex biological matrices, the indirect method is useful for adding highly sensitive fluorescence tags, which are most effective because of its sensitivity and selectivity (Haginaka 2002). No studies were found that describe this procedure in environmental matrices.

2.4.2.4.2 Direct Chiral Separation

The direct method of chiral separation uses chiral stationary phases (CSP) or chiral mobile phase additives (Aboul-Enein 2001; Gubitz et al. 2001; Haginaka 2002). In the three-point interaction model, three simultaneous interactions must take place between the stationary phase and the analyte with at least one being stereoselective for separation to occur (Gubitz et al. 2001; Pirkle et al. 1983). Two of the three interactions are possible by either enantiomer but the presence or absence of the third interaction, which is not in the same plane as the other two, allows the chiral selector to differentiate between the two isomers (Pirkle et al. 1989b). This model does not require all three interactions to be attractive (Pirkle et al. 1989b). Steric repulsive interactions are often involved with one or more bonding interactions to produce chiral recognition (Pirkle et al. 1989b).

Conformation is important for chiral recognition. The shape of the enantiomer affects the elution order and less chiral resolution is found in systems that do not have conformational control of the analyte system (Pirkle et al. 1983). The important key to chiral resolution is the choice of a suitable chiral selector. Enantioselective separation is the result of interactions between the many chiral centers of the CSP and the chiral analyte (Glowka 2002). Enantioselective separation is useful for both preparative purposes and the analysis of enantiomers in standard samples and pharmaceutical preparations, and is preferred because it does not require any derivitization steps (Aboul-Enein 2001; Haginaka 2002). The most useful and popular chiral stationary phases, discussed below, are polysaccharides, cyclodextrins and their derivatives, Pirkle-type phases and macrocyclic antibiotics (Aboul-Enein 2001).

2.4.2.4.3 Polysaccharide Chiral Stationary Phases

The Okamato group introduced polysaccharides chiral stationary phases in 1984 by coating cellulose and amylose derivatives onto pre-treated silica (Aboul-Enein 2001). The versatility, durability and loading capacity of polysaccharides make them are one of the most popular chiral selectors (Chen et al. 2005). They are used in such commercial columns as the Chiralpak AD-RH, available from Daicel, which has a stationary phase consisting of amylose tris-(3,5-dimethylphenylcarbamate) (Fig. 2.4). The differential binding of enantiomers is believed to result from attractive forces such as H-bonding, hydrophobic interactions and dipole-dipole interactions although the mechanism of chiral discrimination has not be satisfactorily elucidated (Aboul-Enein 2001). The polar ester and carbamate groups are the main chiral adsorbing sites on cellulose and amylose derivatives (Aboul-Enein 2001). The introduction of substituents in the phenyl groups can affect the resolving ability of the CSP, although chiral recognition also depends on the fit of the symmetric portion of the analyte into a chiral cavity or channel in the stationary phase (Aboul-Enein 2001). Polar interactions and π-π interactions between the phenyl group of the CSP and aromatic groups of an analyte are important for chiral recognition on polysaccharide chiral stationary phases (Gubitz et al. 2001).

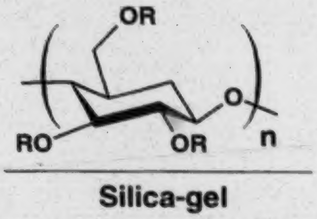
Ye et al. (2002) reported memory effects on the Chiralpak AD, used with normal phase solvents, which depended on the use of mobile phase additives. Extensive washing was required to return the column to control or pre-additive behaviour when mobile phase additives were used (Ye et al. 2002). Because prior exposure to mobile phase additives will affect the performance of an amylosic column, variable results may be obtained from a polysaccharide chiral column depending on its use history (Ye et al. 2002). No further information could be found regarding memory effects in reverse phase mode.

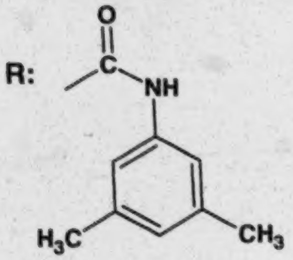
2.4.2.4.4 Cyclodextrin Chiral Stationary Phases

Cyclodextrins (CDs) are cyclic oligosaccharides with six, seven or eight D-(+)-glucose units linked by α-1,4 bonds that form α, β and γ CDs (Fig. 2.4). CDs have been used to resolve the enantiomers of profen drugs such as ibuprofen, ketoprofen and fenoprofen (Ammazzalorso et al. 2005; Choi et al. 2000; Glowka 2002; Haynes et al. 1998; Rozou et al. 2005; Zarizycki et al. 2002). Structurally, cyclodextrins consist of a hollow truncated cone with a hydrophobic inner cavity and a hydrophilic rim (Ammazzalorso et al. 2005). Derivitization can be used to modify both the depth of the cavity and the solubility of the CDs (Gubitz et al. 2001). The different physical properties of the CDs result from differences in their glucose subunits (Glowka 2002). Derivitization of CDs at the 2, 3 or 6 positions allows different compounds to be enantioresolved. Enantiomers are primarily separated based on the inclusion of the bulky hydrophobic group of an analyte into the cavity on the CD (Aboul-Enein 2001; Gubitz et al. 2001; Lipkowitz 2001). Lateral interactions, such as dipole-dipole interactions or hydrogen bonds between the hydroxyl groups at C-2 and C-3 at the upper rim of the cyclodextrin and a hydrophilic part near the stereogenic center of the analyte are also important for chiral recognition (Aboul-Enein 2001; Gubitz et al. 2001). It is also believed that the chiral recognition mechanism depends on hydrophobic interactions, steric repulsion and the mobile phase mode and the organic modifiers used to enhance the solubility of analytes (Aboul-Enein 2001; Gubitz et al. 2001). Water molecules occupy the CD cavity in aqueous solution, even though it is energetically unfavourable (Aboul-Enein 2001). This energy is gained back and acts as a driving force when polar analytes are added to displace the water molecules from the cavity (Aboul-Enein 2001). As with the Chiralpak stationary phase, memory effects were also observed using cyclodextrins stationary phases in polar organic mode (V. McGuffin, personal communication). Changes in retention mechanism and isotherm type from these memory effects are believed to be responsible for the peak shape changes seen during the equilibration process.

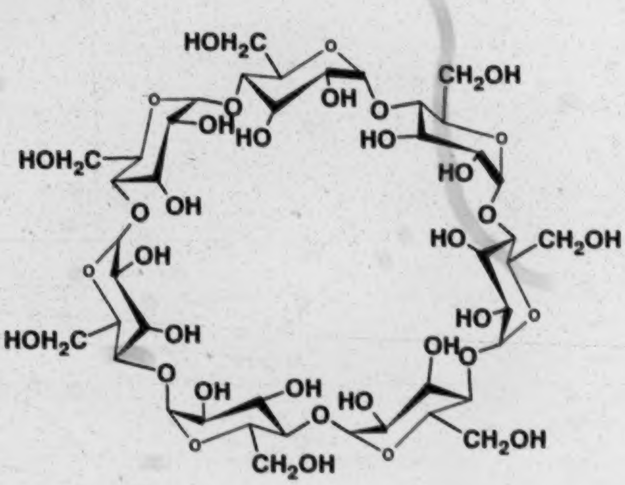
Fig 2.4 Chiral Stationary Phase Structures

Chiralpak AD-RH:
amylos tris (3,5-dimethylphenylcarbamate)

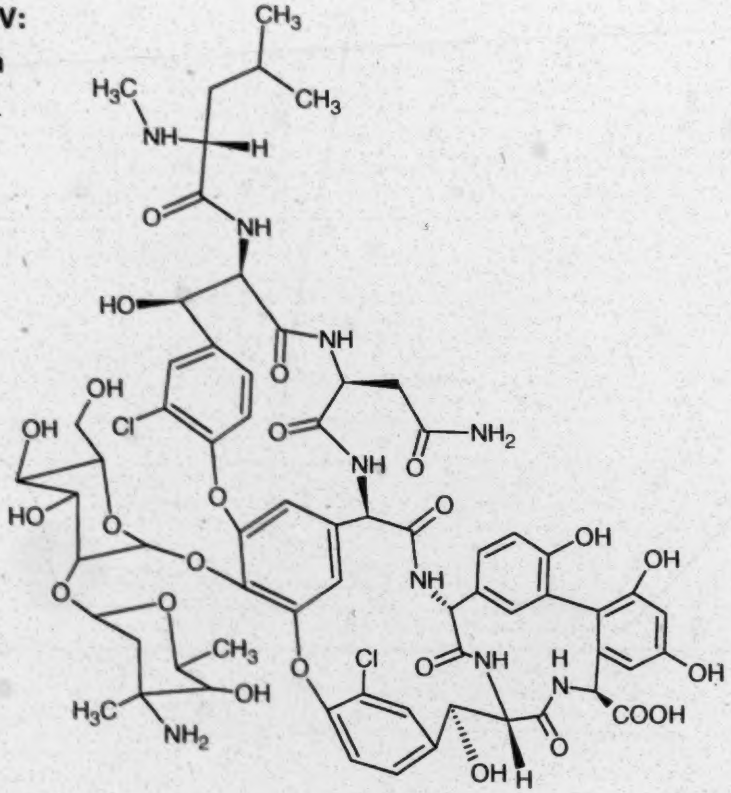

OR
RO
OR
O
n
Silica-gel


R:
O
C
NH
H_3C
CH_3

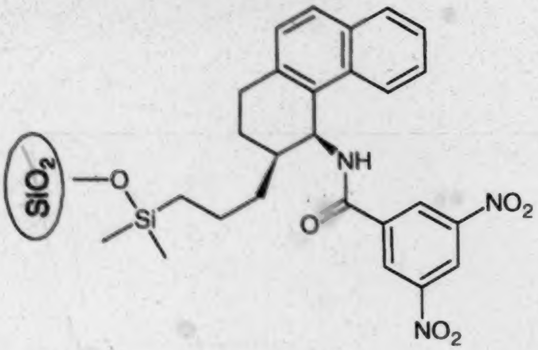
Cyclodextrin:
β–cyclodextrin


HOH_2C
CH_2OH
OH
HO
O

Chirobiotic V:
vancomycin



Whelk-O 1:
(3R,4R)-4-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene


SiO2
O
Si
NH
O
NO2
NO2

The addition of acetic acid or triethylamine sped up the equilibration of methanol from previous conditions involving acetonitrile, however, many days were required for the removal of acidic and basic additives.

2.4.2.4.5 Pirkle-Type Chiral Stationary Phases

The Whelk-O 1 chiral LC column from Regis has a Pirkle-type chiral selector of (3R, 4R)-4-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydro-phenanthrene (Fig. 2.4). Its chiral recognition model is based on π-π interactions, dipole stacking and hydrogen bonding (Gubitz et al. 2001; Lipkowitz 2001; Pirkle et al. 1983; Snyder et al. 2005). Unlike cyclodextrins, Pirkle-type phases do not form inclusion complexes. They are robust, with better reproducibility than polysaccharide based CSP and are well characterized with highly predictable chromatographic behaviours (Chen et al. 2005; Lipkowitz 2001). A model proposed by Wainer and Doyle suggest "face-to-face" recognition (Fig. 2.5), in which the dipoles associated with amide groups will align in an antiparallel manner and cause solute molecules to "stack" onto the most accessible face of the CSP, usually the carboxamide (Pirkle et al. 1989a). In this model, the carboxylic acid moieties are oriented in the same direction (Pirkle et al. 1989a). Because of the stacking behaviour of acylated amines onto the CSP, the alkyl "tail" of the acyl group must intercalate into the bonded phase and be directed towards the silica support (Pirkle et al. 1983). The steric interactions between the alkyl tail and silica that result from this would be unfavourable (Pirkle et al. 1983). A longer tail would lead to more severe interactions, a decrease in the separation factor, α, and less contribution of this model to the overall retention process

(Pirkle et al. 1983). This model has been used to describe the chiral recognition of amides made from amines lacking π-basic substituents (Pirkle et al. 1989a). Pirkle and McCune have proposed a "head-to-tail" model (Fig. 2.5) in which transient adsorbates are formed through the π-π interactions of the CSP and the analyte (Pirkle et al. 1989a; Pirkle et al. 1983). Pirkle et al. (1989) suggest that the structural features of the analyte determine the relative contributions made by two competing chiral recognition processes. During the development of Pirkle-type CSPs, Pirkle et al. (1983) discovered enhanced chiral recognition resulted from an increase in either the π basicity of an aryl group or in the size of an alkyl substituent while an increase in the length of the alkyl "tail" of an acyl group would decrease chiral recognition. Electron-donating substituents on the anilide moiety increased selectivity while electron-withdrawing substituents decreased selectivity (Pirkle et al. 1989a). As the basicity of the carbonyl oxygen is reduced, retention and chiral recognition decreased substantially (Pirkle et al. 1983). In addition to a decreased π basicity of the aryl system, inclusion of additional polar functional groups that might interact with the chiral stationary phase as well as changes in mobile phase composition may be able to affect the balance between competing chiral recognition mechanisms on Pirkle-type phases (Pirkle et al. 1983).

2.4.2.4.6 Vancomycin Chiral Stationary Phases

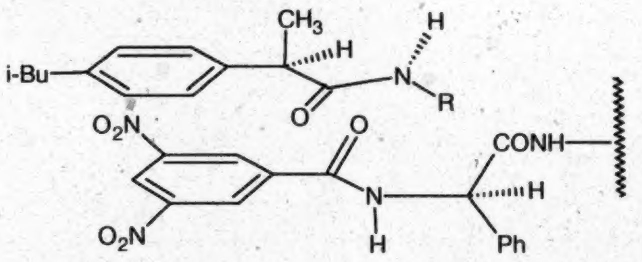
The Chirobiotic V from Astec has a vancomycin chiral selector. It is a bonded macrocyclic antibiotic, amphoteric glycopeptide (Fig. 2.4) with 18 chiral centers, three hydrophobic pockets and five aromatic ring moieties (Chen et al. 2005;

√

Shen et al. 2002). The CSP has multiple interactions with chiral analytes due to the numerous stereogenic centers and functional groups characteristic of macrocyclic antibiotics (Gubitz et al. 2001). The characteristic basket shape and carbohydrate moiety are a result of the glycopeptide fused macrocyclic rings (Gubitz et al. 2001). The hydrophobic basket may allow inclusion of hydrophobic analytes (Gubitz et al. 2001). The CSP has broad selectivity in normal phase, reverse phase and polar organic mode because several distinct enantiomer recognition mechanisms work in close proximity (Chen et al. 2005; Shen et al. 2002). A number of different interactions such as hydrogen bonds, dipole stacking, ionic interactions, π-π interactions and steric repulsions are believed to be responsible for chiral recognition (Gubitz et al. 2001; Shen et al. 2002). For normal phase solvents, π-π and dipole interactions are dominant, while inclusion complexation and H-bonding are favored for reversed-phase conditions (Shen et al. 2002). Since ionization of both analyte and chiral selector are pH dependant, buffer pH is an effective experimental parameter for selectivity (D'Orazio et al. 2005; Pehourcq et al. 2001). For example, at pH 5.0, ketoprofen will be negatively charged but the vancomycin stationary phase will be positively charged and electrostatic interactions are possible (Pehourcq et al. 2001). Although pH should not affect the stereoselective recognition, it may contribute to the non-stereoselective interactions mentioned above in the three-point interaction model. The Chirobiotic V is most stable when run over the pH range of 4.0-7.0 (D'Orazio et al. 2005).

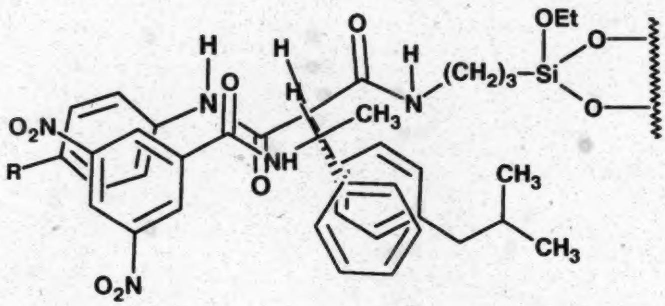
Fig. 2.5 Chiral Recognition Models

Wainer-Doyle "Face-to-Face" model:


CH3
H
H
i-Bu
N
R
O2N
O
O
CONH
N
H
O2N
H
Ph

Representation of the Wainer-Doyle chiral recognition mechanism

Head-to-Tail Diple Stacking chiral recognition model:


OEt
O
H
H
H
O
Si
O
(CH2)3
N
CH3
O2N
R
NH
O
CH3
CH3
O2N

Representation of the Head-to-Tail dipole stacking model

2.4.3 Detectors

2.4.3.1 UV-VIS and Fluorescence Detection

There are many types of detectors available for HPLC analysis, including fluorescence, UV-VIS and mass spectrometry. Fluorescence detectors are sensitive, often more than an order of magnitude greater than absorbance detectors, and are selective (Rouessac et al. 2000). However, this detection source requires the presence of an extended π electron system, like those found in aromatic and highly conjugated species. As such, fluorescence detectors have been used in the analysis of pharmaceuticals and natural products (Hermening et al. 2000; Martinez et al. 1999; Vlase et al. 2005). For example, gemfibrozil has been analyzed using an excitation wavelength of 300 nm and an emission wavelength of 380 nm (Hermening et al. 2000). β-Blockers only weakly absorb light in the UV region and are not well analyzed using these methods (Al-Ghannam 2005). Compounds without native fluorescence can be analyzed through the use of derivatives. Fluorescent derivatives can be formed using reagents such as dansylchloride, rhodamine B and 2',2'-dichlorofluoresceine and allow the detection of primary and secondary amines, amino acids and phenols (Skoog et al. 1998; Vickrey 1983). Information regarding the fluorescence and UV/VIS detection of pharmaceuticals in biological samples, such as urine and plasma, is relatively easy to find (Hermening et al. 2000; Martinez et al. 1999; Mistry et al. 2001; Vlase et al. 2005). However, for environmental samples, extensive clean up procedures are required before fluorescence and UV/VIS detection to remove potential interferences and information is harder to find. Santos et al. (2005) did report detection of diclofenac, ibuprofen, ketoprofen, naproxen, carbamazepine and caffeine in WWTP influent and effluent samples. They cite the expense related to MS systems and the ready availability of HPLC-diode array and fluorescence detectors as the reason this study was conducted using HPLC with diode array and fluorescence detectors (Santos et al. 2005). Limits of quantification ranged from 6.2-320 and 3.0-160 ng/mL for influent and effluent WWTP samples, respectively (Santos et al. 2005). It is worth noting that these concentrations are at least three orders of magnitude higher than those found on LC-MS/MS (ng/L), as discussed above in Section 2.3.3. Although not studied in this thesis, fluorescence has been used in the detection of fluoroquinolone antibiotics in wastewater effluents as a confirmation of their presence while LC-MS was used for quantification (Nakata et al. 2005). However, concentration limits used for fluorescence detection were not given. Although not environmental samples, metoprolol showed a limit of quantification of 0.5 ng/mL using HPLC-fluorescence detection in plasma samples (Mistry et al. 2001). Atenolol, metoprolol and propranolol showed limits of detection of 19 ng/mL, 16 ng/mL and 3 ng/mL, respectively, in pure samples using a fluorometer (Martinez et al. 1999).

Pharmaceuticals have commonly been analyzed using UV/VIS (Franceshi et al. 2005; Lam et al. 2004; Sun et al. 2003). Ketoprofen, for example, has been measured using wavelengths of 233 nm and 261 nm (Dvorak et al. 2004; Ozlu et al. 2005). If separation is not great enough, a wavelength must be chosen that may not be optimal for one of the analytes and the results may not accurately reflect the amount of analyte present. Additionally, the mobile phase used for UV/VIS detection must possess very little absorption in the wavelength range of interest so the compounds of interest can be detected without having their signal swamped out by the solvent. Although UV/VIS and fluorescence have been used in drug analysis, they do not exhibit the sensitivity and selectivity required by the complex matrices found in environmental analysis. Neither UV/VIS nor fluorescence can match the efficiency or speed of mass spectrometry (Tiller et al. 2002). For example, capillary electrophoresis with UV detection has typical detection limits of ~1 μg/mL, which is 1000 times less sensitive than is required for environmental samples (Nakata et al. 2005). Although not environmental samples, ketoprofen was detected in gel and solution form using HPLC-UV/VIS with detection limits of 0.436 μg/mL and 0.303 μg/mL, respectively (Ozlu et al. 2005). Atenolol in pure drug samples had a detection limit of 0.1–0.3 μg/mL using a spectrophotometer (Al-Ghannam 2005). In a phosphate buffered solution, naproxen and ketoprofen had limits of detections of 0.3 ng/mL and 0.25 ng/mL, respectively (Zakeri-Milani et al. 2005). Since these are much cleaner matrices than in environmental samples, it is expected the LOD in environmental matrices will be higher.

2.4.3.2 Mass Spectrometry

Mass spectrometry (MS) is one of the most widely applicable analytical tools and is able to provide elemental composition and structural information. Mass spectrometers function as mass analyzers to separate ions based on their mass-to-charge ratios, m/z. MS has a number of advantages over fluorescence and UV-VIS detectors, including the ability to monitor quickly selected mass ions with enhanced signal-to-noise (S/N), that gives MS high specificity, sensitivity and speed (Annesley 2003). When analyte specific masses or product ions are monitored, interferences that are apparent using UV detection are not observed (Annesley 2003). Some misconceptions have arisen for MS because of the specificity achieved, including the belief that chromatographic separation can be minimized and that specimen cleanup can be minimized or eliminated (Annesley 2003). Lack of separation can lead to ion suppression while specimen cleanup is necessary to reduce matrix effects.

2.4.3.2.1 Electrospray Ionization (ESI)

Two of the most commonly used atmospheric pressure ionization sources used in MS are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Electrospray ionization (ESI) and APCI are complimentary techniques and each has characteristic features give different fragmentation patterns. ESI was first introduced in 1984 and has become very important in the analysis of biomolecules (Skoog et al. 1998). A fused-silica inner capillary and a stainless-steel outer capillary are used to introduce sample and the nitrogen gas used in the nebulization of the sample. Ionization results from a high electric field applied to the capillary needle as the sample is pumped through and is nebulized into a fine spray of droplets. The charged droplets will shrink due to evaporation of the solvent and charge repulsions resulting from the increased charge density will cause sputtering of desolvated ions into the MS source. Electrospray is well suited for direct sample introduction from HPLC and CE columns. Any non-volatile modifiers added to the mobile phase to improve separation might negatively affect ion source performance by altering the surface concentration of analytes in the charged droplet (Chen et al. 2005). ESI is more susceptible to ion suppression than APCI and chromatographic methods involving high water content should be avoided when using ESI because it can decrease ionization efficiency (Desai et al. 2004). ESI is not compatible with normal phase solvents because solvents such as hexane do not support the formation of ions and is a possible explosion hazard due to the high voltage of the electrospray needle (Desai et al. 2004).

2.4.3.2.2 Atmospheric Pressure Chemical Ionization (APCI)

Atmospheric pressure chemical ionization (APCI) involves a heated nebulization quartz tube that forms a gas-vapour mixture from the column eluent. A corona discharge needle ionizes a reagent gas consisting of solvent vapour that is ionized and will transfer the charge to the gaseous analyte (Chen et al. 2005). APCI, like ESI, is considered not suitable for normal phase LC conditions due to the possible explosive hazard due to the presence of solvents such as hexane (Chen et al. 2005). However, APCI does not work well for highly polar and ionic analytes. Generally, it works well for non-polar, volatile and semi-volatile molecules that do not work well with ESI, such as PAHs, PCBs and triglycerides. It has been reported that APCI suffers less from matrix effects than does ESI (Hsieh et al. 2001; Hsieh et al. 2002; King et al. 2000; Mei et al. 2003). A study by Mei et al. (2003) found there were also differences between instruments. They report the APCI interface was actually more susceptible to matrix effects when using a Micromass Quattro Ultima mass spectrometer, while there was no difference between the APCI and ESI mode using a Sciex API 3000 (Mei et al. 2003).

2.4.3.2.3 Triple Quadrupole and Linear Ion Trap Mass Spectrometers

Quadrupole mass spectrometers are the most common type of MS in use. Compared to other types of mass spectrometers, they have high scan speeds and are more compact, less expensive and more rugged (Skoog et al. 1998). Quadrupole mass spectrometers consist of four parallel cylindrical rods with opposite rods connected to a DC source. The DC potential helps to focus the ions towards the center of the quads. Radio frequency AC potentials are applied 180° out of phase to each pair of rods. A positive ion will be attracted to the initially negatively charged rods but when the charged on the rods changes, the ion will be repelled from those rods and attracted to the other pair. This continues as the ion travels through the quads as the ion follows a narrowing "corkscrew" path. Mass spectra are obtained by increasing the AC and DC potentials while keeping their values in a constant ratio. Only a small range of m/z will pass through the quads, with ions of all other m/z striking the quads and being converted to neutral molecules. Quadrupoles can resolve ions with mass differences of only one mass unit. A triple quadrupole has three quadrupoles placed in linear sequence that allows tandem MS, or MS/MS, to be performed. The first quad (Q1) acts as described above. The second quadrupole (Q2) has only a DC potential applied to it and focuses ions travelling through it. Parent ions are fragmented to their daughter ions by a collision gas in Q2. The third quadrupole (Q3) is used to analyze the daughter ions in the same manner as described for Q1. The QTrap 2000 from Applied Biosystems is a triple quad MS/MS that also can act as a linear ion trap. When operated in trap mode, additional potentials are applied to the ends of Q3 that prevent the trapped ions from exiting the trap until the RF potential on Q3 is ramped high enough for the ions to cross the exit barrier potential.

2.4.4 Ionization Suppression

Matrix effects are an important consideration when performing analytical measurements. Analytes extracted from a complex matrix may show a different analytical response than an analyte extracted from a simple matrix because the composition of sample extracts and the presence of interfering ions have proven to have a major influence on analyte signal (Antignac et al. 2005; Brooks et al. 2005). Steps should be taken to minimize or compensate for matrix effects such as improving sample extraction and cleanup or preparing calibration curve standards in matrices identical to the samples of interest (Brooks et al. 2005). Use of standard addition eliminates matrix-effect concerns by comparing the response ratios of both a spiked and unspiked replicate of the sample in question (Brooks et al. 2005). Internal standards are an important part of chromatographic analysis because they help compensate for signal irreproducibility that may lead to inaccurate results. They are best used over a limited retention window and should be structurally similar to the target analytes and elute close to the target. Use of internal standards avoids the use of the time-consuming standard addition method. Many isotopically labelled pharmaceutical internal standards are available, such as D_{10}-carbamazepine, D_3-nicotinic acid methyl ester and D_6-salbutamol (Calamari et al. 2003; Zhu et al. 2005). Internal standards are used in a similar manner to standard addition but do not require both a spiked and unspiked sample to ratio. A common matrix effect found in MS is ion suppression and it is probably one of the main difficulties in LC/MS. Ion suppression can affect detection capability, repeatability and accuracy (Antignac et al. 2005). Less volatile compounds such as salts, ion-pairing agents like NH_4TFA and drug metabolites can change the efficiency of droplet formation or evaporation, which can then affect the amount of charged ion in the gas phase that reaches the detector (Annesley 2003).

2.4.4.1 Characteristics of Ionization Suppression

High variability from one sample to another with different influences between compounds is the main characteristic of ion suppression. A critical situation may result from different degrees of suppression between analyte and internal standard (IS) (Antignac et al. 2005). If the analyte is suppressed more than the IS, the analyte concentration could be underestimated and lead to a false negative conclusion (Antignac et al. 2005). However, if the IS is suppressed more than the analyte, overestimation of analyte concentration could result in a false positive result (Antignac et al. 2005). This is why use of an IS appropriate to the target analytes is needed to balance the disturbance of the analyte signal by an equivalent disturbance of the IS signal (Antignac et al. 2005). Isotopically labelled ^{13}C- or ^{2}H-labelled compounds make ideal internal standards because they are structurally identical to the target analyte and will co-elute with the analyte. They greatly reduce the extent of signal variability seen for the analyte and thereby improve the repeatability of analytical measurements (Antignac et al. 2005). According to Annesley et al. (2003), more polar molecules are more susceptible to ion suppression and higher mass molecules will suppress the signal of smaller molecules. Analytes eluting in the solvent front in reverse phase LC are the highly polar and non-retained compounds while those at the end are the strongly retained compounds (Antignac et al. 2005; Hernando et al. 2004). Antignac et al. (2005) recommend shifting analyte retention times away from the solvent front and the end of the run because these two areas are more affected by interferences and ion suppression. Hernando et al. (2004), however, suggest increasing retention time generally decreases the signal suppression resulting from non-specific matrix effects associated with sample nature and the ionization techniques used.

2.4.4.2 Causes of Ionization Suppression

Although the exact mechanism by which ion suppression works in LC-MS is unknown, there are a few proposed mechanisms. Antignac et al. (2005) propose that the presence of high concentrations of interfering compounds can increase the viscosity and surface tension of the droplets produced during ESI and APCI. This increase reduces the ability of the target analytes to reach the gas phase. The transfer of analytes into the gas phase is also limited through co-precipitation of the analyte with non-volatile materials such as macromolecules (Antignac et al. 2005). The second mechanism proposed by Antignac et al. (2005) involves competition between analytes and interfering compounds regarding the maximum ionization efficiency of the analysis technique. The maximum total concentration of both analytes and interfering compounds allowing efficient ionization of small organic molecules by ESI is 10^{-5} M (Antignac et al. 2005). It is believed the mechanism of ion suppression will be different for ESI (liquid phase ionization) and APCI (gas phase ionization) due to differences in the phase of ionization (Antignac et al. 2005). Additionally, negative mode ionization is generally considered to be more specific and therefore believed to be less subjected to ion suppression (Antignac et al. 2005).

2.4.4.3 Consequences of Ionization Suppression

A main consequence of ion suppression is the reduction in detection capability due to a decrease in analyte signal. Significant variability in the degree of ion suppression from one sample to another can affect the repeatability of analytical measurements. Orthogonal source spray design may be able to improve transmission of analyte ions while simultaneously discarding unwanted species that may interfere with the analytes (Antignac et al. 2005). Changes in the MS method may be advantageous because the rest of the developed analytical procedure, including sample preparation and chromatographic conditions, need not be modified (Antignac et al. 2005). The only definitive way to avoid ion suppression is through improved sample preparation and purification because it limits the presence of interfering compounds in the final extract (Antignac et al. 2005). Potential interfering compounds include ionic species such as salts, highly polar compounds and various organic molecules such as carbohydrates, lipids and metabolites as well as plastic and polymer residues, ion-pairing agents and buffers.

2.4.4.4 Solutions to Ionization Suppression

Many additives have been used to counteract ion suppression, including tri-n-butylamine and 2-(2-methoxyethoxy) ethanol (2-MEE) to increase signal intensity (Quitana et al. 2004; Yamaguchi et al. 1999). Instead of using TFA for ion-pairing, other weaker acids such as acetic acid, formic acid or hexafluorobutyric acid should be investigated (Annesley 2003). However, the acetate anion may give signal suppression in an electrosprayed droplet due to ion competition between the analyte and acetate anion (AcO^-) (Yamaguchi et al. 1999). The typical solvents used for reverse phase LC are methanol and acetonitrile. The high volatility of these solvents mean they evaporate faster than water and form droplets containing a high percentage of water and AcO^- that are not able to evaporate into smaller droplets (Yamaguchi et al. 1999). It is unfortunate that the mobile phases needed for chromatographic resolution are often not adequate for ESI (Yamaguchi et al. 1999).

Chapter 3: Analytical Methods and Environmental Results

Chapter 3.1 Introduction

Pharmaceuticals in the environment are an emerging concern because large quantities are produced and consumed each year, and the occurrence and fate of these compounds is not fully understood (Daughton et al. 1999; Metcalfe et al. 2003a). There is concern that a constant influx of pharmaceuticals into the environment may result in a continuous environmental presence that is not predicted by their degradability (Lam et al. 2004). Many pharmaceuticals have been detected at low levels (to ng/L) in the environment due to human excretion, veterinary usage, improper disposal and resistance to degradation during the wastewater treatment process (Andreozzi et al. 2003; Buerge et al. 2003; Buser et al. 1998b; Buser et al. 1998c; Calamari et al. 2003; Comoretto et al. 2005; Debska et al. 2004; Heberer 2002; Huggett et al. 2003; Kolpin et al. 2002; Loffler et al. 2003; Metcalfe et al. 2003a; Metcalfe et al. 2003b; Miao et al. 2004; Petrovic et al. 2005b; Richards et al. 2004; Roberts et al. 2005; Till 2005; Zuccato et al. 2005).

Pharmaceuticals are designed to cause biological effects in both humans and animals at low concentrations. As a result, it is important to understand their environmental fate and to know whether the pharmaceuticals are accumulating or being removed from the environment (Comoretto et al. 2005). Although the environmental concentrations may not be acutely toxic, a few studies have investigated the effects that chronic exposure to low concentrations may have on aquatic organisms (Brooks et al. 2003; Cleuvers 2004; Emblidge et al. 2005;

Gagne et al. 2005; Hernando et al. 2004; Lin et al. 2005; Mimeault et al. 2005; Schwaiger et al. 2004). Fluoxetine was found at levels >0.1 ng/g in fish residing in a municipal effluent-dominated system and was found to decrease the growth of *Pseudokirchneriella subcapitata* (algae) at 14 µg/L (Brooks et al. 2005; Brooks et al. 2003). Naproxen showed average chronic toxicity values of 27.9 to 36.3 mg/L on *P. subcapitata*, 0.40-0.62 mg/L on *Brachionus calyciflorus* (rotifer) and 0.11-0.63 mg/L on *Ceriodaphnia. dubia* (crustaceans) (Isidori et al. 2005). Cleuvers (2004) determined half-maximal effective concentrations (EC_{50}) on the order of mg/L for diclofenac, ibuprofen and naproxen, respectively, on *Daphnia magna* (water flea) immobilization (Cleuvers 2004). Although these concentrations are much higher than the concentrations of pharmaceuticals currently found in the environment (ng/L), Mimeault et al. (2005) have shown bioconcentration of gemfibrozil in the blood plasma of goldfish (bioconcentration factor of 113) at environmentally relevant concentrations. This led to a 50% decrease in the plasma testosterone levels of the fish with the conclusion that pharmaceuticals have the potential to cause deleterious effects on aquatic life (Mimeault et al. 2005).

Although the environmental occurrence of pharmaceuticals is a growing field, there has been little work done on the occurrence of chiral pharmaceuticals in the environment to date. The chirality of these drugs is an important issue that must be taken into consideration because of the potentially different pharmacological, pharmacokinetic and toxicological effects that the individual isomers may have on biological systems (Pehourcq et al. 2001). The enantiomers may have different effects on aquatic organisms than are observed in the intended target organisms. One enantiomer often has a higher therapeutic effect, or may have negative side effects (Lees et al. 2003; Yang et al. 2005). Because of this potential, some drugs are marketed as a single enantiomer, such as S-(+)-naproxen (Lees et al. 2003; Shen et al. 2002; Yang et al. 2005). Certain chiral pharmaceuticals, such as ketoprofen, fenoprofen and ibuprofen, have also demonstrated an inversion mechanism whereby the body can biotransform the inactive isomer into the active isomer (Castro et al. 2000; Igarza et al. 2002; Moraes de Oliveira et al. 2005b; San Martin et al. 2002; Sevoz et al. 1999). This inversion can lead to a larger dose of the active isomer than would be expected based on the administered dose. Buser et al. (1999) have determined the enantiomer ratios (ER) of ibuprofen in German wastewater and surface water samples. The ERs were calculated using:

$$ER = \frac{[\text{S Enantiomer}]}{[\text{R Enantiomer}]} \qquad (3.1)$$

where a racemic mixture has an ER of 1. The ERs ranged from 5.5 to 8.0 in influent, and from 0.9 to 2.0 in effluents (Buser et al. 1999). German surface waters had ibuprofen ERs ranging from 0.7 to 2.1 (Buser et al. 1999). Although not a pharmaceutical, the chiral herbicide mecoprop was found to have an ER of 2.5 in Lake Sempachersee in Germany (Buser et al. 1998b). Fono et al. (2005) found racemic enantiomer fractions (EF) for propranolol (0.50 ± 0.02) in five WWTP influents and EFs ranging from 0.30 to 0.44 after secondary (biological) treatment. The EF is calculated using:

$$EF = \frac{E1}{E1 + E2} \tag{3.2}$$

if the enantiomer elution order is unknown, where E1 is the first eluting enantiomer and E2 is the second eluting enantiomer, or:

$$EF = \frac{(+)}{(+) + (-)} \tag{3.3}$$

where (+) is the (+)-enantiomer and (-) is the (-)-enantiomer if the enantiomer elution order is known. An EF value of 0.5 is racemic. These results clearly showed non-racemic values for chiral molecules in German environmental and WWTP samples, meaning stereoselective degradation is occurring in these environmental samples. However, little is currently known about the environmental fate and effects of most pharmaceuticals.

The low environmental concentrations and the complicated matrices in which pharmaceuticals occur demands sensitive and selective techniques for their quantification. Although gas chromatography has been used for environmental pharmaceutical analysis, it often requires an added derivitization step to ensure the pharmaceuticals are volatile enough for analysis (Boyd et al. 2004; Brooks et al. 2005; Buerge et al. 2003; Buser et al. 1999; Scheytt et al. 2005). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is able to avoid this cumbersome procedure and results in faster analysis times and easier analytical procedures. Thus, it is the technique of choice for environmental analysis of drugs due to its high sensitivity and ability to provide unambiguous target identification. The stereoisomer composition of drugs is best quantified by chiral LC-MS/MS. However, many of the chiral stationary phases available are for normal phase LC and thereby not applicable to MS analysis (Desai et al. 2004; Okamato et al. 1994; Perrin et al. 2002). Additionally, many buffers used for environmental and pharmaceutical analysis are non-volatile and should not be used with MS (Dvorak et al. 2004; Lam et al. 2004; Novakova et al. 2005; Ozlu et al. 2005). Although chiral separations are often used in pharmaceutical production and for separation from biological samples, they have generally not been applied to trace environmental analysis (Mistry et al. 2001; Moraes de Oliveira et al. 2005a; Moraes de Oliveira et al. 2005b; Shen et al. 2002; Welch et al. 1997). Many pharmaceutical separations are monitored using UV detectors that do not have the sensitivity needed for environmental analysis (Lam et al. 2004; Ozlu et al. 2005; Sun et al. 2003). Thus, it can be seen that selective and sensitive methods are needed for the determination of chiral pharmaceuticals in environmental samples.

In this work, we present the analytical methods used in the determination of achiral and chiral pharmaceuticals in surface waters and wastewaters. A solid phase extraction method, using Waters Oasis HLB cartridges, was used to extract various classes of pharmaceuticals that are frequently detected in environmental samples, including non-steroidal anti-inflammatories, lipid regulators, beta-blockers, selective serotonin reuptake inhibitors and stimulants. Three chiral LC columns, which have shown applicability in the determination of the target pharmaceuticals in reverse-phase chiral LC were used to analyze chiral and achiral pharmaceuticals in the Alberta aquatic environment (Moraes de Oliveira et al. 2005a; Shen et al. 2002; Welch et al. 1997). The goal of this research was to develop methodologies using LC-MS/MS for the analysis of such compounds to help understand the environmental fate of these emerging pollutants.

Chapter 3.2 Experimental

3.2.1 Chemicals and materials

Achiral indomethacin, diclofenac and clofibric acid, *S*-(+)-naproxen, and racemic ibuprofen, ketoprofen, and fenoprofen were purchased from ICN Biomedical (Aurora, OH, USA). Gemfibrozil, caffeine, *S*-(+)-ketoprofen, *S*-(+)-ibuprofen, *S*-(-)-atenolol, *R*-(-)-fluoxetine and racemic atenolol, propranolol and metoprolol were purchased from Sigma-Aldrich (Oakville, ON, Canada). Racemic fluoxetine and (+)-levobunolol were purchased from Toronto Research Chemicals (North York, ON, Canada). Labelled D_{10}-carbamazepine and D_6-2,4-D were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The structures, CAS numbers and drug classes are found in Figure 2.1. HPLC grade methanol was used to prepare working solutions of all analytes because of insolubility in acetonitrile and water at high concentrations. The working solutions were stored in amber vials at 4°C and brought to room temperature before use. Serial dilutions containing all analytes were prepared in methanol at 100, 75, 50, 25, 10, 1, 0.1 and 0.01 ng/mL. All calibration standards contained 50 ng/mL of each internal standard, chosen based on their structural similarity to the analytes. The deuterium-labelled pesticide D_6-2,4-D was used as the negative mode standard based on the phenoxy-group and the carboxylic acid moiety that all of the negative mode targets contain. The deuterium-labelled anti-convulsant D_{10}-carbamazepine was used as a positive mode internal standard because of the aromatic rings that are similar to those of fluoxetine and caffeine. D_{10}-carbamazepine was not a suitable internal standard for the β-blockers, so the unlabelled β-blocker (+)-levobunolol was used as the positive mode internal standard due to its structural similarity to metoprolol, atenolol and propranolol. A deuterated form of this drug was unavailable at the time of analysis. However, (-)-levobunolol is the active isomer used in eye drops in the treatment of glaucoma. Because it is used as an eye drop, the drug is not likely to make it into the environment compared to drugs taken internally and the analysis of blank samples proved that levobunolol was not natively present at detectable levels. To further ensure this internal standard would not be found in the environmental samples, the (+)-isomer which is not present in pharmaceutical preparations was used.

HPLC grade methanol and acetonitrile were ordered from Fisher Scientific (Ottawa, ON, Canada). Nanopure water was obtained from a Nanopure Ultrapure Water System (Barnstead/Thermolyne, Dubuque, IA, USA). Formic acid, acetic acid, triethylamine, ammonium acetate, phosphoric acid, potassium phosphate monobasic, ammonium trifluoroacetate, oxalic acid and ammonium nitrate were purchased from Fisher Scientific (Ottawa, ON, Canada) and were of analytical grade (> 98% purity) and used as supplied. Triethylammonium acetate (TEAA) was prepared by acidifying a triethylamine solution with acetic acid to the desired pH. All mobile phases were filtered through 0.22 μm nylon filters before use.

3.2.2 Instrumentation and Equipment

Initial method development was performed using a Hewlett Packard (Agilent, Mississauga, ON, Canada) 1050 quaternary pump with an autosampler and UV/VIS detector, and an Agilent 1100 binary pump and autosampler with an MSD single quadrupole mass spectrometer. They will not be discussed further. Two triple quadrupole instruments were used for quantitative analysis. The first instrument used consisted of an HP1100 binary pump and an Agilent 1100 autosampler coupled to a Micromass Quattro (Waters, Mississauga, ON, Canada) triple quadrupole mass spectrometer equipped with a Z-spray electrospray interface. Instrument control, data acquisition and evaluation were done with MassLynx NT Software (version 3.5). The second instrument was an HP1100 binary pump with HP1100 autosampler, Harvard syringe pump, inline degasser, chiller and column heater coupled to a Sciex QTrap 2000 (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray™ electrospray source. Instrument control, data acquisition and evaluation were done with Analyst Software (version 1.4.1). All triple quadrupole experiments were performed using electrospray ionization (ESI) and multiple reaction monitoring (MRM). An inline filter was used to prevent particulate matter from reaching the analytical columns.

Three reverse phase chiral columns were used in this investigation. A 150mm × 4.6mm Chiralpak AD-RH (5 µm particle size, Daicel, Chiral Technologies, West Chester, PA, USA) amylose column was used with a 10 mm × 4 mm Chiralpak AD-RH guard cartridge. It was used in both positive *and negative MS mode, and demonstrated simultaneous chiral and achiral* separation. A 250 mm × 4.6 mm (R,R)-Whelk-O 1 (5 μm particle size, Regis Technologies, Morton Grove, IL, USA) Pirkle-type column was used in both positive and negative mode and demonstrated simultaneous chiral and achiral separation. A 250 mm × 4.6 mm Chirobiotic V (5 μm particle size, Advanced Separation Technologies, Inc., Whippany, NJ, USA) vancomycin based column was being used with positive ionization to demonstrate chiral and achiral separation. The Whelk-O 1 and Chirobiotic V both used a 10 mm × 3 mm nitrile guard cartridge.

Solid-phase extraction was performed using 3 mL Waters Oasis HLB Sample Extraction Cartridges (Waters, Mississauga, ON, Canada) containing 60 mg of sorbent. Prevail Extract-Clean columns (C_{18}) from Alltech Associates, Inc. (Deerfield, IL, USA) were also briefly investigated. All extracted samples were passed through Whatman 0.22 μm PTFE syringe filters (13 mm, Whatman Inc., Clifton, NJ, USA) before analysis.

3.2.3 Chromatographic Conditions

The chromatographic conditions used are shown in Table 3.1. All runs used a 25 μL injection and the flow was split 1:1 post-column. Each column required different conditions to achieve chiral separation. Both the Chiralpak AD-RH and Whelk-O 1 used different gradients and runtimes for positive and negative modes, discussed further in Results and Discussion. During analysis on the QTrap LC-MS/MS, samples were kept at 4°C and promptly refrigerated after use to prevent decomposition. On the Micromass Quattro LC-MS/MS, samples remained at room temperature during analysis but were refrigerated after use.

3.2.4 Mass Spectrometry Conditions

Initial MS/MS development and some quantification were done using a Micromass system while quantitation was performed with the Sciex system. The optimized conditions for the Micromass Quattro are shown in Table 3.2. The mass spectrometry (MS) conditions used for the QTrap 2000 are shown in Table 3.3. All parameters were optimized using direct infusion of 1 µg/mL solutions of each drug in HPLC grade methanol. Full scan spectra were used to confirm the parent/molecular ion. The $[M+H]^+$ ion was used for the positive mode targets while the $[M-H]^-$ ion was used for negative mode targets. The most intense daughter fragment ion for each compound was chosen for quantitative analysis. The conditions used on the Quattro were as follows: capillary voltage ± 2.2 eV, gas cell pressure 1×10^{-3} mbar, source temperature 120°C, desolvation temperature 350°C. Nitrogen was used as both nebulization gas and desolvation gas at flow rates of 90 and 375 L/hr, respectively. The Quattro was not used in the analysis of environmental samples and will not be discussed further. The QTrap 2000 was used for quantification of environmental samples. The declustering potential (DP) is used to break apart ion clusters as they enter the MS and the collision cell entrance potential (CEP) is used to focus the ions entering the collision cell. Both were optimized for each parent ion. Daughter ion scans were run and the base peaks were used as the daughter ions.

Table 3.1 Chromatographic Conditions used in this Study

	Chiralpak AD-RH	Chirobiotic V (1)	Chirobiotic V (2)	Whelk-O 1
Positive Mode	40:60 to 57:43 12(0)	90:10	30:70	60:40 to 90:10 15(1)
Solvents	AcN/ 0.05% formic acid	MeOH/ 0.05% TEAA approx. pH 5.0	AcN/ 2 mM NH_4OAc + 0.1% formic acid	MeOH/ 0.05% formic acid
Runtime	12 minutes	20 minutes	15 minutes	16 minutes
Flow rate	0.5 mL/min	1 mL/min	0.5 mL/min	0.5 mL/min
Internal Standard	D_{10}-carbamazepine	(+)-levobunolol	n.d.	D_{10}-carbamazepine
Negative Mode	40:60 to 49:51 13(4) to 57:43 7(0)	N/A	N/A	60:40 to 69:31 11.5(0) to 90:10 5(8.5)
Solvents	AcN/ 0.05% formic acid			MeOH/ 0.05% formic acid
Runtime	24 minutes			25 minutes
Flow rate	0.5 mL/min			0.5 mL/min
Internal Standard	D_6-2,4-D			D_6-2,4-D
Chiral analytes Resolved	fenoprofen ibuprofen	metoprolol atenolol propranolol	fluoxetine	ketoprofen ibuprofen

N/A indicates no samples were run using this ionization mode
n.d. means there was no appropriate internal standard at the time of this analysis

Multiple reaction monitoring (MRM) was used to optimize the collision energy (CE) and collision cell exit potential (CXP) for each parent to daughter transition (Table 3.3). Mass spectrometer parameters also were optimized. The ion spray voltage (IS), curtain gas (CUR), nebulizing gas (GS1), heater gas (GS2) and temperature (TEM) of GS2 were optimized, along with the probe height and electrode position. The QTrap 2000 used the following optimized conditions: curtain gas setting of 25; collisionally activated dissociation (CAD) gas setting of 5; nebulizing gas (GS1) and heater gas (GS2) settings of 20 and 60, respectively; GS2 temperature 400°C; ion energy 5500 V in positive mode and –4500 V in negative mode. Nitrogen was used as both the curtain gas and CAD gas, while zero air was used as the nebulizing gas and heater gas.

Table 3.2 Optimized Micromass Quattro MRM Conditions

Mode	Drug	MRM Transition	Collision Voltage (eV)	Cone Voltage (eV)
Positive	Fluoxetine	310.3 > 148.0	15	17
	Caffeine	194.9 > 137.9	18	22
	D_{10}-carbamazepine	247.0 > 204.0	10	52
Negative	D_6-2,4-D	225.0 > 167.0	14	21
	Clofibric acid	212.9 > 127.0	15	19
	Ketoprofen	253.1 > 208.9	15	20
	Naproxen	228.9 > 170.0	15	11
	Fenoprofen	241.3 > 196.8	15	17
	Diclofenac	293.9 > 250.0	15	18
	Ibuprofen	205.3 > 161.0	12	17
	Indomethacin	356.3 > 311.9	12	18
	Gemfibrozil	249.4 > 120.9	12	19

Table 3.3 Optimized QTrap 2000 MRM Conditions

Mode	**Drug**	**MRM Transitions**	**Declustering Potential (DP)**	**Entrance Potential (EP)**	**Collision Cell Entrance Potential (CEP)**	**Collision Energy (CE)**	**Collision Exit Potential (CXP)**
Positive	**Fluoxetine**	310.3 > 148.0	30	10	12	14	2
	Caffeine	194.9 > 137.9	42	10	15	24	2
	Metoprolol	268.2 > 133.1	45	10	10	36	3
	Atenolol	267.2 > 145.0	52	10	10	35	2
	Propranolol	260.2 > 182.9	45	10	10	25	3
	D_{10}-carbamazepine	247.0 > 204.0	81	10	14	31	6.5
	(+)-levobunolol	292.0 > 236.0	76	10	14	23	2.5
	D_4-nicotinic acid ethyl ester	156.0 > 128.0	57	10	15	20	3.5
Negative	**D_6-2, 4-D**	225.0 > 167.0	-51	-10	-14	-20	0
	Clofibric acid	212.9 > 127.0	-32	-10	-14	-22	-1
	Ketoprofen	253.1 > 208.9	-17	-10	-14	-16	-4
	Naproxen	228.9 > 170.0	-17	-10	-11	-22	-4
	Fenoprofen	241.3 > 196.8	-17	-10	-14	-16	-2
	Diclofenac	293.9 > 250.0	-26	-10	-14	-18	-2
	Ibuprofen	205.3 > 161.0	-32	-10	-26	-22	-4
	Indomethacin	356.3 > 311.9	-36	-10	-35	-22	-2
	Gemfibrozil	249.4 > 120.9	-35	-10	-14	-36	-4

3.2.5 Sample Collection and Extraction

Water samples were collected in 4 L amber glass bottles. All glassware used during collection, sample extraction and standard preparation was cleaned using Contrad 70 liquid detergent (Decon Laboratories Inc., King of Prussia, PA, USA) and rinsed with HPLC-grade solvent and nanopure water before use. All water samples (wastewater, surface water and nanopure water) were filtered to remove suspended particulate matter using 0.45 µm glass fiber filters, precombusted at 450°C for 4 hours. Extraction volumes of 100 mL and 500 mL were used for wastewater influents and effluents, respectively, while 1000 mL were extracted for surface and nanopure waters. Solid phase extraction (SPE) was performed using Oasis HLB cartridges (3 mL, 60 mg) from Waters (Waters, Mississauga, ON, Canada) with no pH adjustment of the samples. The cartridges were conditioned by passing 2 × 3 mL filtered HPLC grade methanol, followed by 2 × 3 mL filtered nanopure water (Nanopure Ultrapure Water System, Barnstead Thermolyne, Dubuque, Iowa). The cartridges were then filled with water and PTFE sippers (Scientific Products & Equipment, North York, Ontario) were used to deliver samples to the cartridges at a rate of 2 mL/min. Cartridges were allowed to dry under vacuum for 15 minutes, and were then centrifuged for five minutes to remove any remaining water. Methanol (6 mL) was added to the cartridges in two portions to elute the target pharmaceuticals. The eluate was passed through a drying column made of anhydrous precombusted Na_2SO_4 and filtered using a Whatman 0.22 µm PFTE syringe filter. Samples were concentrated to 1 mL using a nitrogen evaporator and 50 µL of a 1 µg/mL internal standard spiking solution was added. Finally, 25 µL were injected for analysis.

3.2.6 Data Analysis and Interpretation

When stereoisomers are not baseline resolved, the peaks need to be deconvoluted before calculating the enantiomer fractions (EF). PeakFit (version 4.0.6.0, Systat Software, Inc., Point Richmond, CA) was used to deconvolute enantiomer peaks during chiral separation. A four parameter Gaussian model was used for the deconvolution (Ulrich et al. 1998; Wong et al. 2001). The standard deviations were determined for all repetitive analyses and a t-test was performed when necessary with a significance level of <0.05. If $P > 0.05$, the values were considered not statistically different. The values are included in the table when possible or in a separate table when not possible.

Chapter 3.3 Results and Discussion

3.3.1 Methods Development

3.3.1.1 Optimization of Chromatographic Conditions

Chromatographic conditions were optimized (Table 3.1) for positive (fluoxetine, caffeine, metoprolol, atenolol and propranolol) and negative (clofibric acid, ketoprofen, naproxen, fenoprofen, diclofenac, ibuprofen, indomethacin and gemfibrozil) mode targets separately. Internal standards were chosen based on structural similarity to the target analytes. The positive mode analytes were quantified using either D_{10}-carbamazepine or (+)-levobunolol as internal standard. D_{10}-carbamazepine could not be seen on the Chirobiotic V and therefore could not be used for quantification of the β-blockers, so (+)-levobunolol was used instead. When the fluoxetine chiral separation was performed on the Chirobiotic V, (+)-levobunolol had not yet been chosen and no internal standard was used. However, no quantification could be performed using that method due to difficulties with the column that prevented reproducible results. Ideally; an isotopically labelled single-enantiomer β-blocker would have been chosen for a new internal standard. However, the only labelled β-blocker readily available at the time was D_9-salbutamol and it was prohibitively expensive. Thus, the decision was made to use an unlabelled β-blocker, levobunolol. Because of the application format and the specific isomer used, it is not likely to be found in the environment. Unspiked WWTP samples were run to ensure levobunolol was not present in any samples, and no levobunolol peak was found in any of them. Because of this, it was decided that (+)-levobunolol would be an appropriate internal standard for the quantification of β-blockers on the Chirobiotic V. The negative mode analytes were quantified using D_6-2,4-D for all analyses.

Three chiral columns were used: Chiralpak AD-RH, Chirobiotic V and Whelk-O 1. Methanol and acetonitrile were tested as organic modifiers and a variety of aqueous buffers were tested. Initial methods development involved phosphate buffers. Although this resulted in good chromatographic separation, phosphate buffers are not appropriate for MS detectors due to a lack of volatility. Pehourcq et al. (2001) used an ammonium nitrate buffer with tetrahydrofuran (THF) as the organic modifier to separate ketoprofen on a Chirobiotic V column with a UV detector. An ammonium nitrate buffer was tested on our column but was not volatile enough for MS analysis and did not result in chiral chromatographic separation. Methanol was used as the organic modifier because THF is not compatible with the PEEK tubing used on the instrument. Chiral separation was seen for fluoxetine in positive mode using 0.05% NH_4TFA in methanol. However, none of the other compounds, including the internal standards, were observed. Trifluoroacetic acid (TFA) is known to cause ionization suppression above 0.1% concentration in positive mode ionization and will cause complete suppression in negative mode ionization (Davis 2005; Shou et al. 2005). This buffer was not used because the lack of internal standard meant fluoxetine could not be quantified. Gradient elution was necessary for shorter analysis times on both the Chiralpak AD-RH and the Whelk-O 1 columns, although different gradients were needed for the different analytes observed in positive and negative mode. Ultimately, acetonitrile and formic acid buffer were chosen for the Chiralpak AD-RH, methanol and ammonium acetate/formic acid buffer were used for the Chirobiotic V and methanol and formic acid buffer were chosen for the Whelk-O 1. Chiral separation was observed in positive mode only on the Chirobiotic V, while it was observed in negative mode only on the Chiralpak AD-RH and the Whelk-O 1. The retention times of each analyte and internal standard on the three chiral columns can be found in Table 3.4.

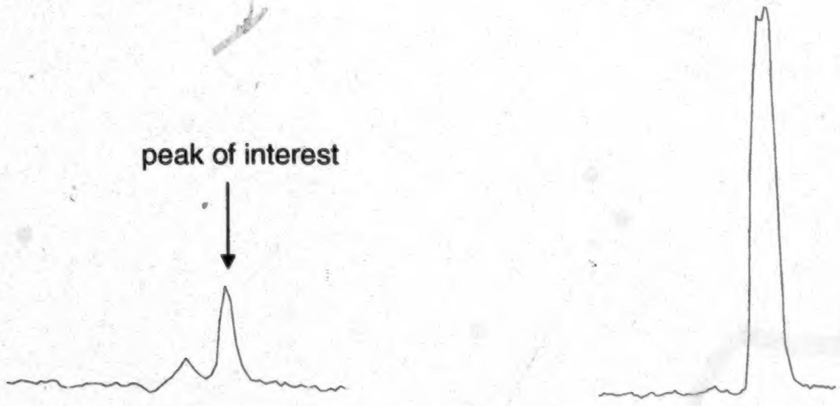
Post-column splitting was used to decrease the flow entering the MS. This decreases the amount of organic compounds that undergo ionization at the same time (Kloepfer et al. 2005). Lower flow rates also result in a decreased droplet size and an increase in surface area (Kloepfer et al. 2005). This results in more efficient electrospray ionization by reducing the amount of analytes and matrix compounds that are competing for desolvation and ionization (Kloepfer et al. 2005). The flow reaching the MS can be changed using different internal diameter and length PEEK tubing for the waste line (Kloepfer et al. 2005).

Table 3.4 Retention Times on Three Chiral Columns
n.d. indicates the analyte was not seen under those conditions

Drugs	Chiralpak AD-RH	Chirobiotic V	Whelk-O 1
Fluoxetine	5.3	n.d.	3.3
Caffeine	7.3	n.d.	12.6
Metoprolol 1	4.4	8.6	n.d.
Metoprolol 2		9.1	n.d.
S-(-)-Atenolol	4.0	11.9	n.d.
R-(+)-Atenolol		12.5	n.d.
S-(-)-Propranolol	4.6	9.5	n.d.
R-(+)-Propranolol		10.0	n.d.
D_{10}-Carbamazepine	11.1	n.d.	10.6
(+)-Levobunolol	4.3	12.01	n.d.
D_6-2,4-D	9.4	n.d.	8.4
Clofibric acid	10.2	n.d.	8.3
Ketoprofen 1	11.5	n.d.	11.7
Ketoprofen 2			11.9
Naproxen	14.6	n.d.	14.9
Fenoprofen 1	14.6	n.d.	10.8
Fenoprofen 2	14.7	n.d.	11.4
Diclofenac	16.1	n.d.	12.5
Indomethacin	20.9	n.d.	n.d.
Gemfibrozil	22.0	n.d.	13.5

The post-column splitting increased the S/N and decreased the ion suppression seen for the analytes (Fig. 3.1). Both total ion current (TIC) signal traces seen in Fig. 3.1 were collected using a 150 ng/mL mixture of fluoxetine, caffeine and D_{10}-carbamazepine in positive mode with either (a) no post-column splitting or (b) post-column splitting. The split was measured by draining the waste line into a graduated cylinder. There was an improvement in S/N of 3-4 times that in the non-split trace (Fig. 3.1 a) and is very evident in the split trace (Fig. 3.1 b).

Figure 3.1 TIC Comparison of a 150 ng/mL mixture containing fluoxetine, caffeine and D_{10}-carbamazepine with (a) no post-column split and (b) with 1:1 post-column split


a) Positive mode TIC with a 20 μL injection volume and no post-column split

b) Positive mode TIC with a 20 μL injection volume and a 1:1 post-column split

The Chiralpak AD-RH has shown excellent achiral separation in addition to the desired chiral separation (Figs. 3.2 and 3.3). Achiral separation is important because co-elution can lead to ion suppression in mass spectrometry (Choi et al. 2001). The achiral separation on the Chirobiotic V was not as well resolved on either the Chiralpak AD-RH or the Whelk-O 1, presumably due to the similarity in the β-blocker structures. However, the concentration of the TEAA buffer as well as the precise pH of the solution is known to cause dramatic differences in the retention and chiral separation of analytes on the Chirobiotic V column (Bosakova et al. 2005). Bosakova et al. (2005) found poor enantioseparation resulted from high concentrations of TEAA (1% TEAA) and that pH varied the stereoselective interactions. Work performed by Sherri MacLeod at the University of Alberta (not shown) at different TEAA buffer pHs showed increases in the analyte retention time as pH was increased and thus demonstrated the importance of accurate solution pH determination when using this column. It is likely that the chiral and achiral resolution on the Chirobiotic V can be improved through strict control of the TEAA concentration and pH, which was not performed during the course of this work.

Fig 3.2 Chiralpak AD-RH positive mode separation showing achiral resolution of fluoxetine and caffeine but lack of resolution of metoprolol, atenolol and propranolol

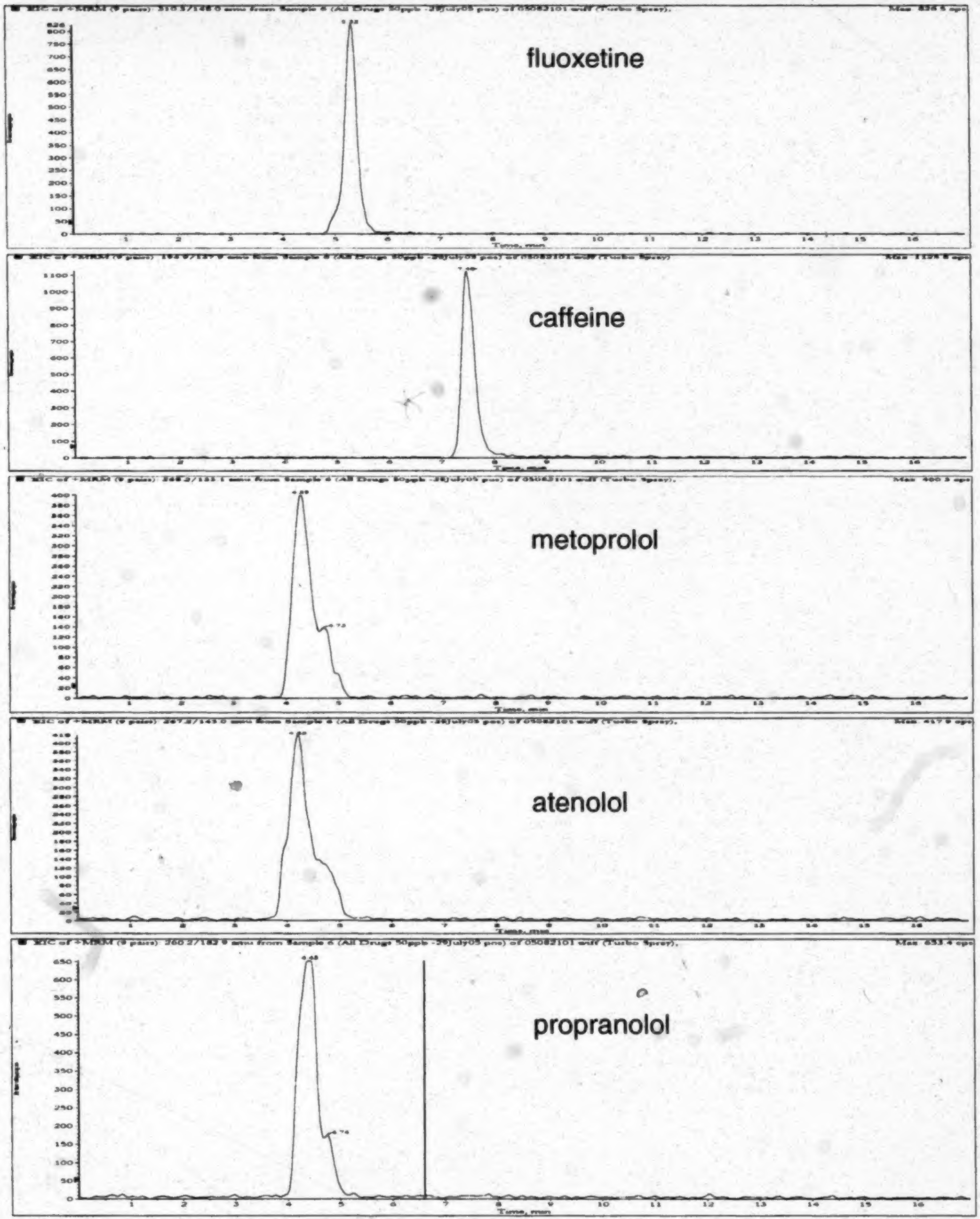
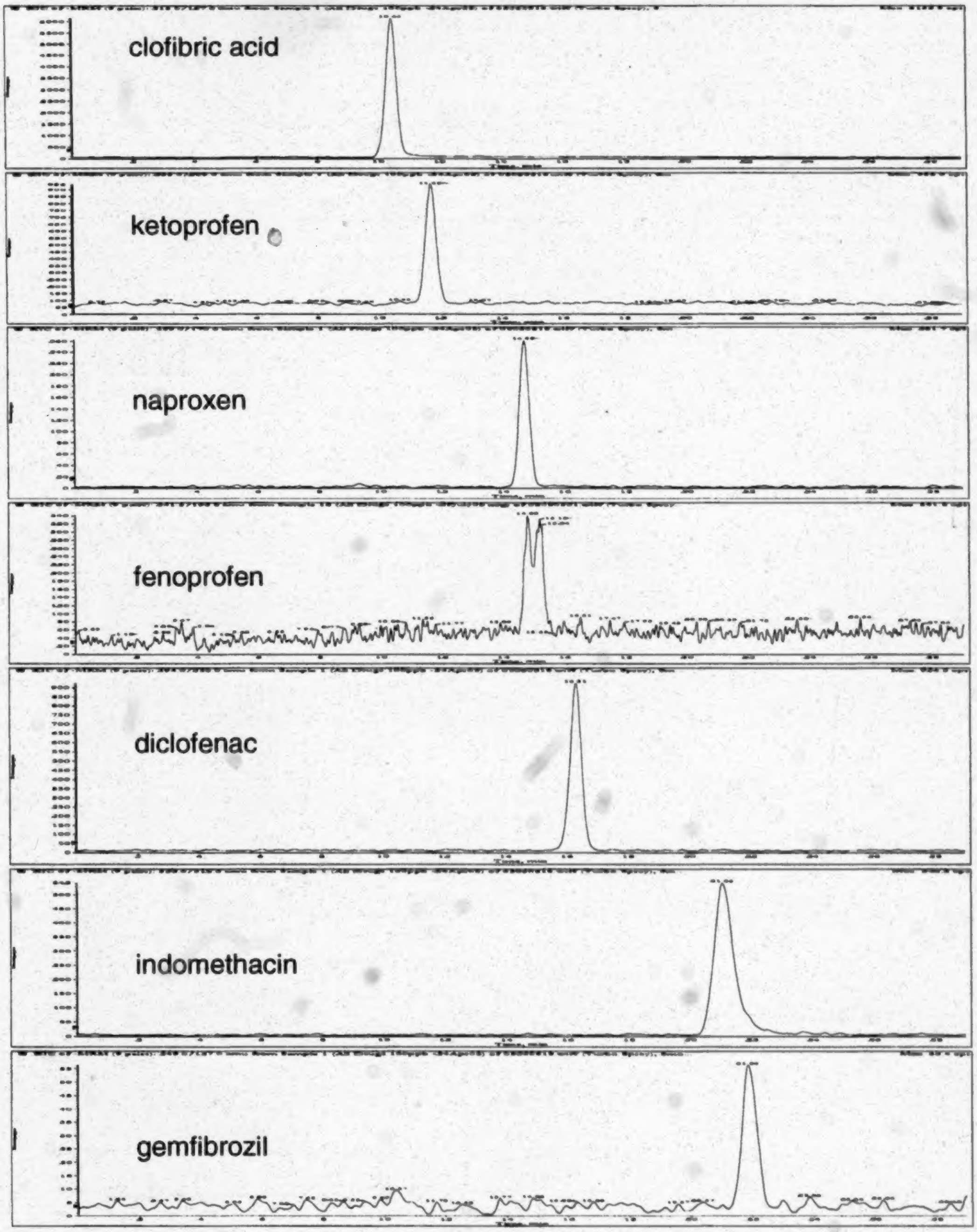


Fig 3.3 Chiralpak AD-RH negative mode separation showing achiral resolution and chiral resolution on fenoprofen



The chiral separations seen on the Chirobiotic V and Chiralpak AD-RH are shown in Figure 3.4 and tabulated in Table 3.5. The resolutions were calculated using Equation 3.4:

$$Rs = \frac{(t_{R_2} - t_{R_1})}{0.5 * (W_1 + W_2)} \qquad (3.4)$$

where t_{R1} and t_{R2} refer to the retention times of the first and second eluting enantiomers, respectively, and W_1 and W_2 are the baseline widths. Metoprolol, atenolol and propranolol separated on the Chirobiotic V with resolutions of 0.7, 0.8 and 0.9, respectively. They were not resolved on either the Chiralpak AD-RH or the Whelk-O 1. Fenoprofen was resolved on the Chiralpak AD-RH with a resolution of 0.6. Ibuprofen initially showed excellent resolution on both the Chiralpak AD-RH (resolution = 2.6) and the Whelk-O 1 (resolution = 3.0) (data not shown). However, it was not separated on the Chirobiotic V for either set of conditions. Difficulties developed in the analysis of ibuprofen (discussed in Section 3.5) that resulted in the inability to measure this drug on the QTrap 2000, and the problem has yet to be resolved. Fluoxetine was resolved only on the Chirobiotic V using ammonium acetate/formic acid buffer. However, these results could not be reproduced on a new smaller 2.1 mm I.D. column that was used in place of the old 4.6 mm I.D. column. This could be due to differences in the buffer composition and pH, as discussed above. Ketoprofen was resolved only on the Whelk-O 1, with a resolution of 0.8. However, the chromatography on the Whelk-O 1 degraded over time to such a point that chiral resolution was lost.

Fig. 3.4 Enantiomer Separation of chiral analytes on Chirobiotic V (metoprolol, atenolol, and propranolol) by positive mode electrospray and on Chiralpak ADRH (fenoprofen) by negative mode electrospray

Note that deconvolution of the resolved peaks indicates they are racemic.

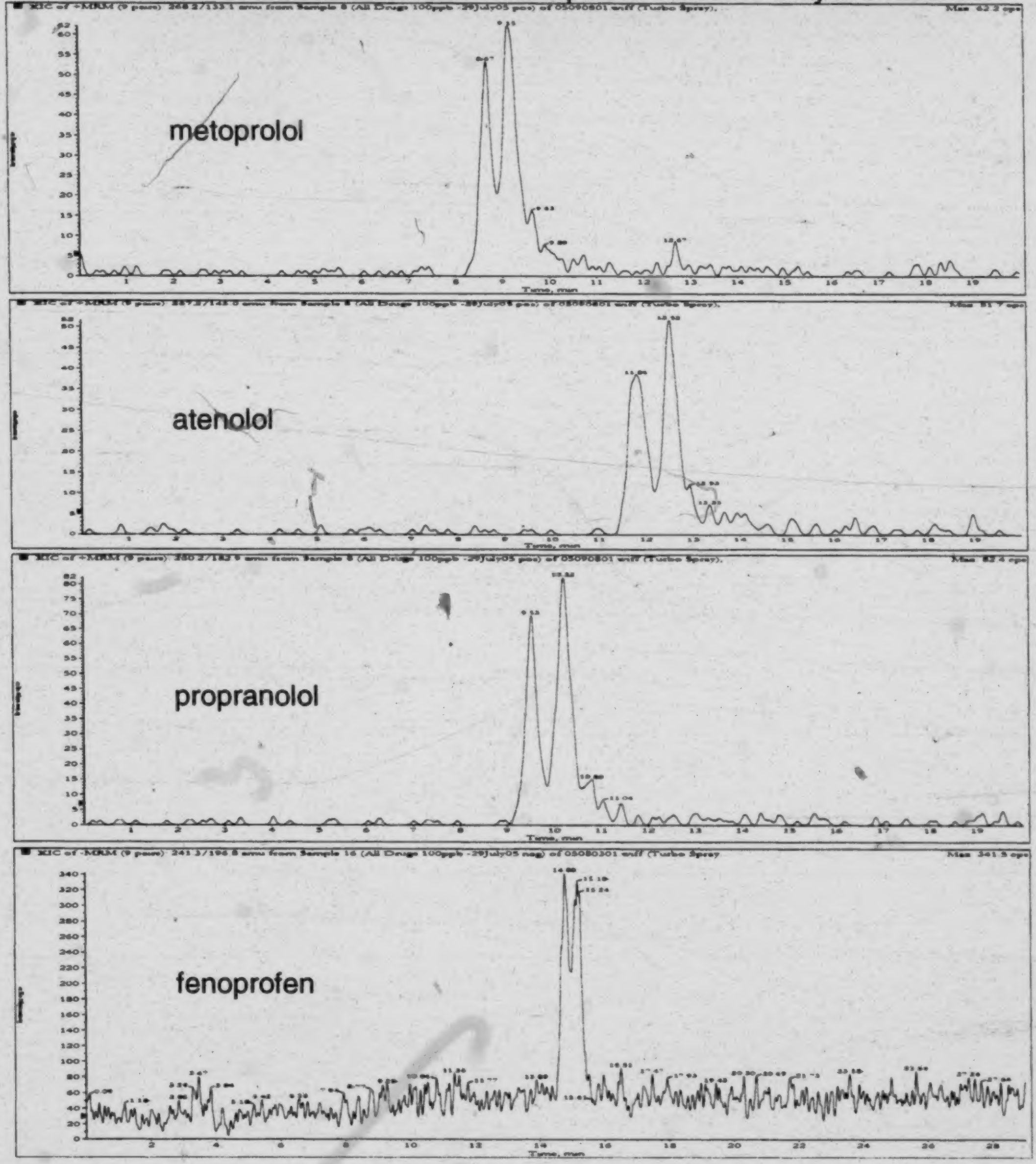


Table 3.5 Resolution Factors for Chiral Analytes on Chiralpak AD-RH and Chirobiotic V in Comparison to Literature Values

Drug	Chiralpak AD-RH	Chirobiotic V	Literature
Fluoxetine	-	-	1.17 [a]
Metoprolol	-	0.7	1.36 [b]
Atenolol	-	0.8	1.66 [b]
Propranolol	-	0.9	1.12 [b]
Ketoprofen	-	-	1.12 [c]
Fenoprofen	0.6	-	14.0 [d]

a = (Shen et al. 2002), b = (D'Orazio et al. 2005), c = (Pehourcq et al. 2001), d = (Haynes et al. 1998)

As mentioned above, Pehourcq et al. (2001) were able to resolve ketoprofen using an NH_4NO_3 buffer and THF, which could not be utilized on the QTrap 2000 due to incompatibility of THF with the tubing used on the instrument and a lack of volatility of the NH_4NO_3 buffer. Perhaps resolution would have been possible if these conditions could have been reproduced. Naproxen is enantiomerically pure, so no enantiomer resolution is applicable. Table 3.5 presents values for chiral resolution in the literature. Fluoxetine had a resolution of 1.17 using a Chirobiotic V column and polar organic mode conditions. D'Orazio et al. (2005) performed nano-LC experiments using a custom-made vancomycin-modified stationary phase and β-blockers. Metoprolol, atenolol and propranolol had resolution factors of 1.36, 1.66 and 1.12, respectively, using methanol and ammonium acetate buffer conditions (D'Orazio et al. 2005). The improved separation is likely due to the use of nano-LC with custom-made columns, compared to our study that used conventional LC and readily- available chiral columns. Ketoprofen was resolved with a resolution factor of 1.12 on a

Chirobiotic V column using an ammonium nitrate buffer and THF on an HPLC-UV instrument (Pehourcq et al. 2001). Due to the incompatibility of these solvents with our LC-MS/MS, the results could not be duplicated and the resolution of ketoprofen was not possible using the Chirobiotic V. Perhaps the electrostatic interactions possible using an ammonium nitrate buffer and THF were not present in our method, which used TEAA and methanol (Pehourcq et al. 2001). If these interactions are not present, it is not surprising that the resolution of ketoprofen was not seen.

3.3.1.2 Optimization of Solid Phase Extraction

Solid-phase extraction (SPE) cartridges were chosen based on literature reports showing their applicability to the target pharmaceuticals. Waters Oasis HLB sample extraction cartridges were selected due to the high percent recoveries they have typically shown in the analyses of the target pharmaceuticals (Castiglioni et al. 2005; Lin et al. 2005; Metcalfe et al. 2003b; Miao et al. 2003; Mimeault et al. 2005; Ollers et al. 2001; Petrovic et al. 2005b; Tixier et al. 2003; Weigel et al. 2004b). During the optimization process, the effects of pH, extraction matrix, cartridge type and elution volume were investigated. It was expected that pH would play a large role in the extraction of pharmaceuticals from water samples by controlling the deprotonation of acidic drugs and the protonation of basic drugs. Ibuprofen, ketoprofen, fenoprofen, clofibric acid, indomethacin, gemfibrozil, diclofenac and naproxen are acidic pharmaceuticals and are expected to be best extracted under acidic conditions while fluoxetine, caffeine, metoprolol, atenolol and propranolol are basic pharmaceuticals and should be best extracted under basic conditions. Table 3.6 shows average percent recoveries at pH 2, 7 and 11 in four matrices: nanopure water; river water; and WWTP influent and effluent. Recoveries improved at higher pH for more complex matrices. This was unexpected for the acidic drugs based on the above hypothesis. However, Oasis HLB cartridges are designed to require no pH adjustment for sample extraction, and the results in Table 3.6 agree. While most studies were performed at pH 2, there are a limited number of studies that did perform SPE using Oasis HLB cartridges at neutral pH for many of the target pharmaceuticals (Miao et al. 2003; Sabbioni et al. 2004; Santos et al. 2005; Weigel et al. 2004a; Weigel et al. 2004b). In nanopure water and river water, there were generally small differences between % recovery at pH 2 and pH 7. Only caffeine showed a large increase in recovery at pH 11 compared to pH 2 (65% at pH 2 vs. 97% at pH 11). Fluoxetine (P = 0.005, Table 3.7), atenolol (P = 0.034, Table 3.7) and diclofenac (P = 0.040, Table 3.7) percent recoveries were statistically different, with fluoxetine showing better recovery at pH 2 and atenolol and diclofenac showing better recovery at pH 7. The remaining analytes were not statistically different based on the t-test values (Table 3.7). The large recoveries seen for fenoprofen in nanopure water (158% to 280%) are likely due to the low resolution of the enantiomers and the difficulties that arise during the integration of such peaks. It can be difficult to reproducibly integrate low, poorly resolved peaks and this may lead to errors in the recoveries. For WWTP influents and effluents, recoveries were generally better at pH 7.

Table 3.6 SPE Percent Recoveries in Four Matrices at Differing Sample pH

N = 3
Nanopure water and river water sample volumes were 500 mL.
WWTP Influent sample volume was 100 mL. WWTP Effluent sample volume was 500 mL
Influent and Effluent values are averages from both Gold Bar and Lac la Biche WWTP
σ is the standard deviation
n/a indicates % recoveries that could not be determined
Native concentrations have been accounted for in these results

Drug	**Nanopure water, % (± σ)**			**River water, % (± σ)**		**WWTP Influent, % (± σ)**		**WWTP Effluent, % (± σ)**	
	pH 2	**pH 7**	**pH 11**	**pH 2**	**pH 7**	**pH 2**	**pH 7**	**pH 2**	**pH 7**
Fluoxetine	71 ± 12	49 ± 9	60 ± 1	39 ± 2	49 ± 10	200 ± 19	292 ± 864	228 ± 36	105 ± 54
Caffeine	82 ± 4	65 ± 10	97 ± 3	64 ± 5	76 ± 6	9070 ± 1420	437 ± 4910	453 ± 123	982 ± 609
Metoprolol	94 ± 24	95 ± 24	50 ± 1	81 ± 7	103 ± 15	26 ± 11	104 ± 34	61 ± 7	59 ± 111
Atenolol	0	71 ± 28	51 ± 1	1 ± 1	98 ± 11	49 ± 2	74 ± 16	54	n/a
Propranolol	89 ± 2	92 ±24	51 ± 1	61 ± 2	88 ± 14	74 ± 1	104 ± 54	88 ± 38	87 ± 32
Clofibric acid	104 ± 6	98 ± 6	59 ± 9	101 ± 5	81 ± 11	55 ± 5	130 ± 22	73 ± 4	59 ± 18
Ketoprofen	108 ± 2	103 ± 7	122 ± 7	123 ± 10	114 ± 9	18 ± 5	187 ± 67	44 ± 6	128 ± 27
Naproxen	118 ± 15	122 ± 22	131 ± 10	102 ± 12	111 ± 27	n/a	n/a	33 ± 18	130 ± 97
Fenoprofen 1	170 ± 27	280 ± 24	124 ± 30	277 ± 18	269 ± 66	n/a	284 ± 54	51 ± 23	148 ± 382
Fenoprofen 2	158 ± 29	248 ± 18	111 ± 14	216 ± 22	227 ± 44	n/a	190 ± 29	29 ± 17	144 ± 462
Diclofenac	101 ± 10	114 ± 9	123 ± 0.3	111 ± 8	111 ± 8	n/a	55 ± 332	n/a	n/a
Indomethacin	103 ± 14	94 ± 14	10 ± 2	58 ± 13	58 ± 13	n/a	264 ± 151	0.3 ± 6	125 ± 121
Gemfibrozil	171 ± 10	164 ± 19	244 ± 16	157 ± 34	157 ± 34	n/a	713 ± 483	104 ± 109	268 ± 305

Table 3.7 T-test values for SPE Recoveries at pH 2 and pH 7

n/a means t-test values could not be calculated due lack of samples or because background levels were too high

Drug	T-test Value		
	Nanopure water	WWTP Influent	WWTP Effluent
Fluoxetine	0.005	0.297	0.005
Caffeine	0.065	0.001	0.112
Metoprolol	0.487	0.001	0.475
Atenolol	0.034	0.049	n/a
Propranolol	0.454	0.003	0.487
Clofibric acid	0.176	1.75×10^{-5}	0.245
Ketoprofen	0.107	2.73×10^{-6}	1.74×10^{-7}
Naproxen	0.390	n/a	0.001
Fenoprofen 1	0.088	n/a	0.092
Fenoprofen 2	0.066	n/a	0.096
Diclofenac	0.040	n/a	n/a
Indomethacin	0.220	n/a	0.001
Gemfibrozil	0.449	n/a	0.051

Caffeine (P = 0.001), metoprolol (P = 0.001), atenolol (P = 0.049), propranolol (P = 0.003), clofibric acid (P = 1.75×10^{-5}) and ketoprofen (P = 2.73×10^{-6}) all showed statistical differences between pH 2 and pH 7 in WWTP influent samples. Fluoxetine did not show a statistical difference. T-tests could not be performed for the remaining WWTP influent samples either because there were not enough samples at pH 2 (N = 1) or because the recoveries could not be determined due to high background levels (Table 3.6). In the WWTP effluents, fluoxetine (P = 0.005), ketoprofen (P = 1.74 x 10^{-7}), naproxen (P = 0.001) and indomethacin (P = 0.001) showed statistical differences between pH 2 and pH 7. Diclofenac could not be determined and the remaining analytes were not statistically different, although gemfibrozil was close (P = 0.051).

Many of the WWTP samples have recoveries that are unreasonably high (> 300%). Additionally, the samples marked "n/a" in Table 3.6 had negative recoveries. Both of these problems are likely due to low spiked concentrations compared to high native concentrations. Ideally, spike and recovery experiments are performed with a spiked concentration much higher than any native concentration to prevent interferences between the concentration added and the concentration already present. In this case, however, the spiked levels (50 ng/mL) were close to or lower than the native concentration of the pharmaceuticals in the sample. Even after accounting for the native concentrations of the pharmaceuticals, this resulted in some recovery calculations that were negative.

In addition to the percent recovery differences, extracts at pH 2 also resulted in messy chromatograms with undesirable interfering peaks that were not seen in higher pH extracts. This was especially true in WWTP samples but was also seen for nanopure water and was largely confined to negative mode analysis, that is, to the organic acids. These interferences are likely due to ionized DOC in the environmental samples that become protonated at pH 2 and are easily co-extracted. There may also be polar compounds or ionic salts eluting early that could account for the peaks seen in the nanopure water samples. The high polarity of these interferences would explain the early elution during reverse phase chromatography.

Only clofibric acid and diclofenac showed a statistical difference between the S/N for the samples at pH 2 and pH 7 (Table 3.8). As such, ion suppression is not expected to be responsible for the chromatographic differences seen. Figure 3.5 illustrates this for a nanopure water sample in negative mode. Large peaks are seen between four and ten minutes in the fenoprofen MRM transition at pH 2. Smaller peaks are also seen in the same range for ketoprofen and naproxen. Such large peaks can make quantification difficult and should be minimized using an appropriate pH. Wastewater influent and effluent samples are shown in Figures 3.6 through 3.9. The WWTP influent sample at pH 2 (Fig. 3.6) shows large undesirable peaks for ketoprofen, fenoprofen and indomethacin. In the case of fenoprofen, the undesirable peaks are significantly larger than the analyte peak (t_R ~ 15 min). The naproxen and diclofenac chromatograms show much smaller but still clearly evident extraneous peaks. When compared to the influent sample at pH 7 (Fig. 3.7), fenoprofen still has the extra peaks but the analyte peak is larger compared to the interferences. The ketoprofen and indomethacin transitions show insignificant interferences compared to the pH 2 extract. The effluent samples at pH 2 (Fig. 3.8) show a similar pattern of interferences. In these samples, naproxen shows a large peak that was not seen in the influents but ketoprofen has very small interference peaks compared to the influent. In the effluent at pH 7 (Fig. 3.9), only fenoprofen shows a significant interference peak but it is not larger than the analyte peak this time. This is an improvement over the pH 2 samples. The Chirobiotic V shows dramatic differences between pH 2 and pH 7 samples as well, as illustrated by Fig. 3.10 and 3.11. The WWTP effluent sample at pH 2 (Fig. 3.10) shows messy, broad peaks that show no chiral separation. Additionally, the atenolol peak is barely above the baseline noise. At pH 7 (Fig. 3.11) however, clear chiral separation can be seen for all three β-blockers and the atenolol peak is clearly seen. Based on these results, pH 7 was chosen for SPE extractions.

Table 3.8 Sample S/N Ratios for 100 ng/mL Mixture of Negative Mode Analytes at pH 2 and pH 7

N = 3

	Signal to Noise (S/N) Ratio		
Drug	**pH 2**	**pH 7**	**T-Test values**
Clofibric Acid	191 ± 23	145 ± 8	0.029
Ketoprofen	42 ± 4	42 ± 2	0.460
Naproxen	28 ± 3	34 ± 6	0.113
Fenoprofen 1	12 ± 3	11 ± 0.5	0.392
Fenoprofen 2	11 ± 2	13 ± 2	0.239
Diclofenac	152 ± 18	188 ± 18	0.035
Indomethacin	42 ± 1	46 ± 5	0.144
Gemfibrozil	34 ± 3	34 ± 6	0.497

Figure 3.5 Chromatograms of 50 ng/mL mixture of negative mode target analytes extracted from nanopure water at pH 2 on Chiralpak AD-RH

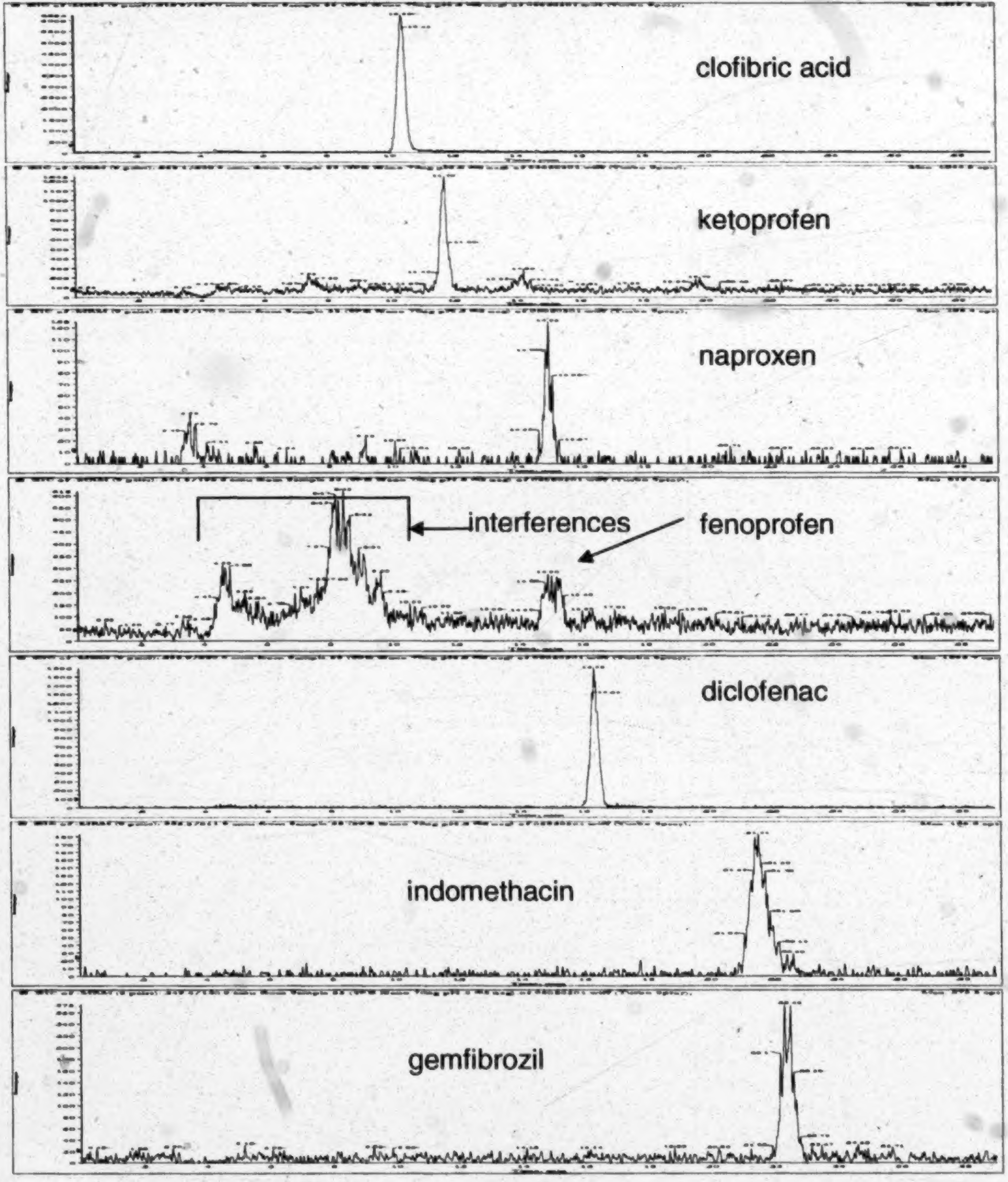


Figure 3.6 Chromatograms of 50 ng/mL mixture of negative mode target analytes extracted from WWTP Influent at pH 2 on Chiralpak AD-RH

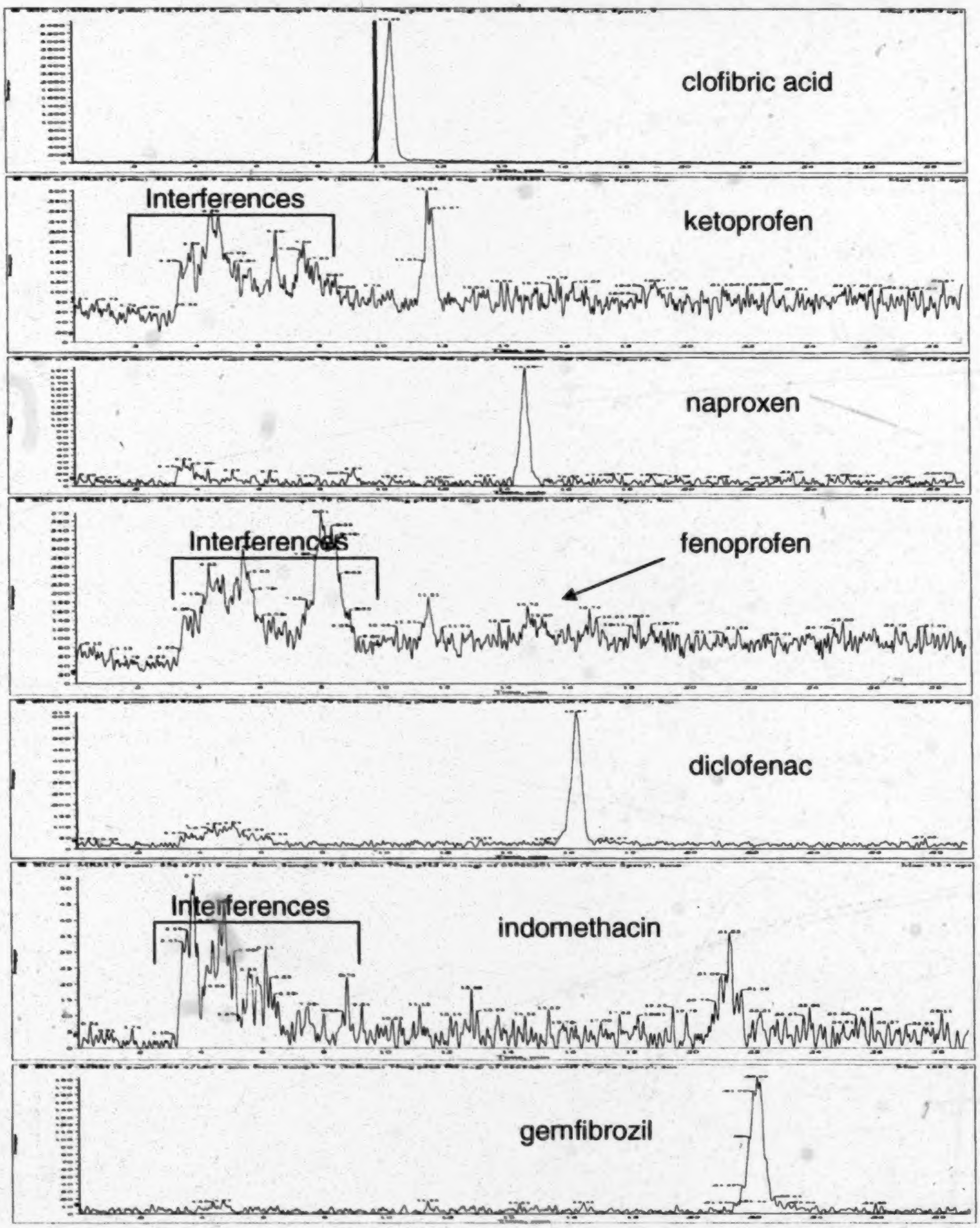


Figure 3.7 Chromatograms of 50 ng/mL mixture of negative mode target analytes extracted from WWTP Influent at pH 7 on Chiralpak AD-RH

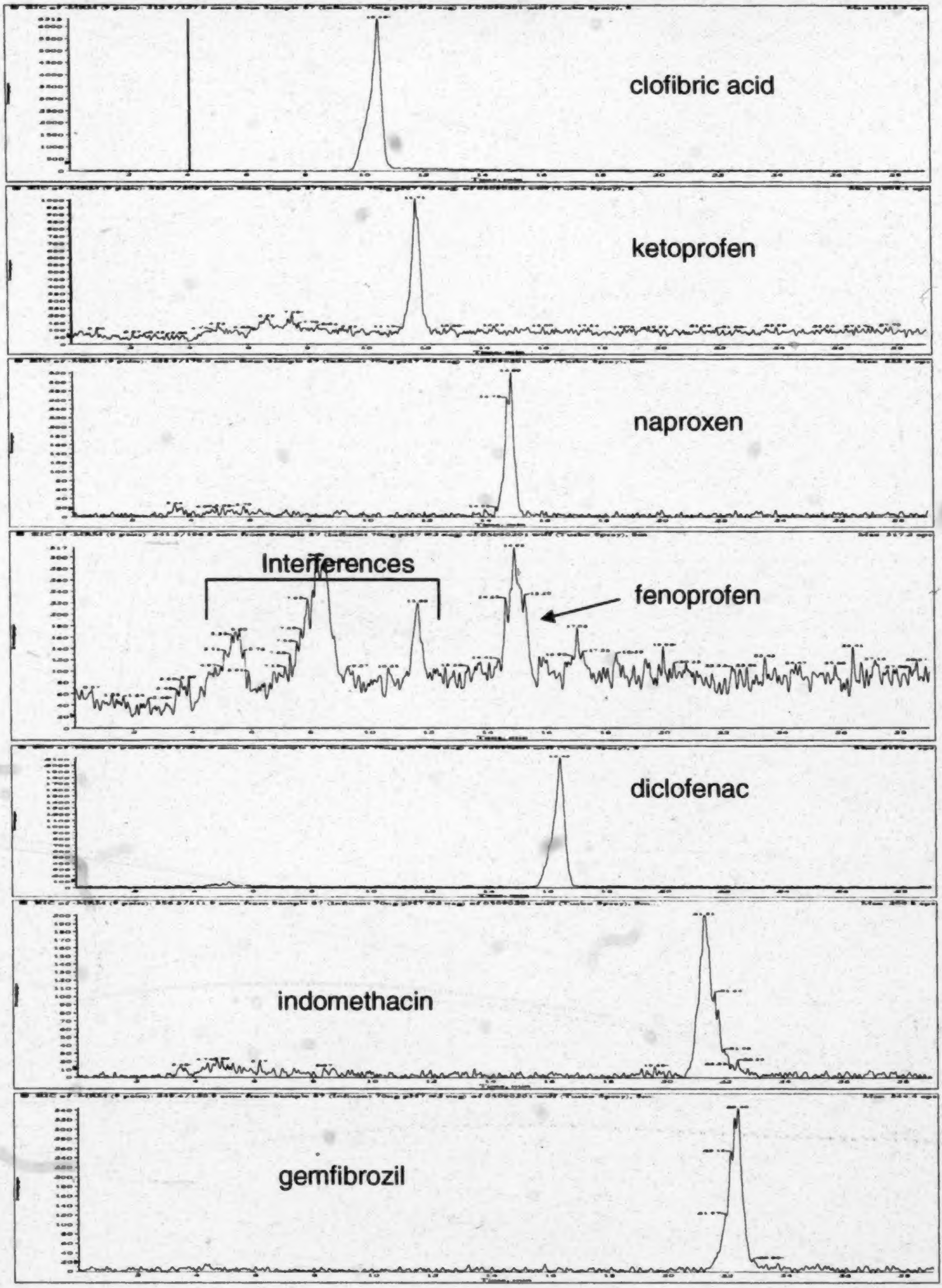


Figure 3.8 Chromatograms of 50 ng/mL mixture of negative mode target analytes extracted from WWTP Effluent at pH 2 on Chiralpak AD-RH

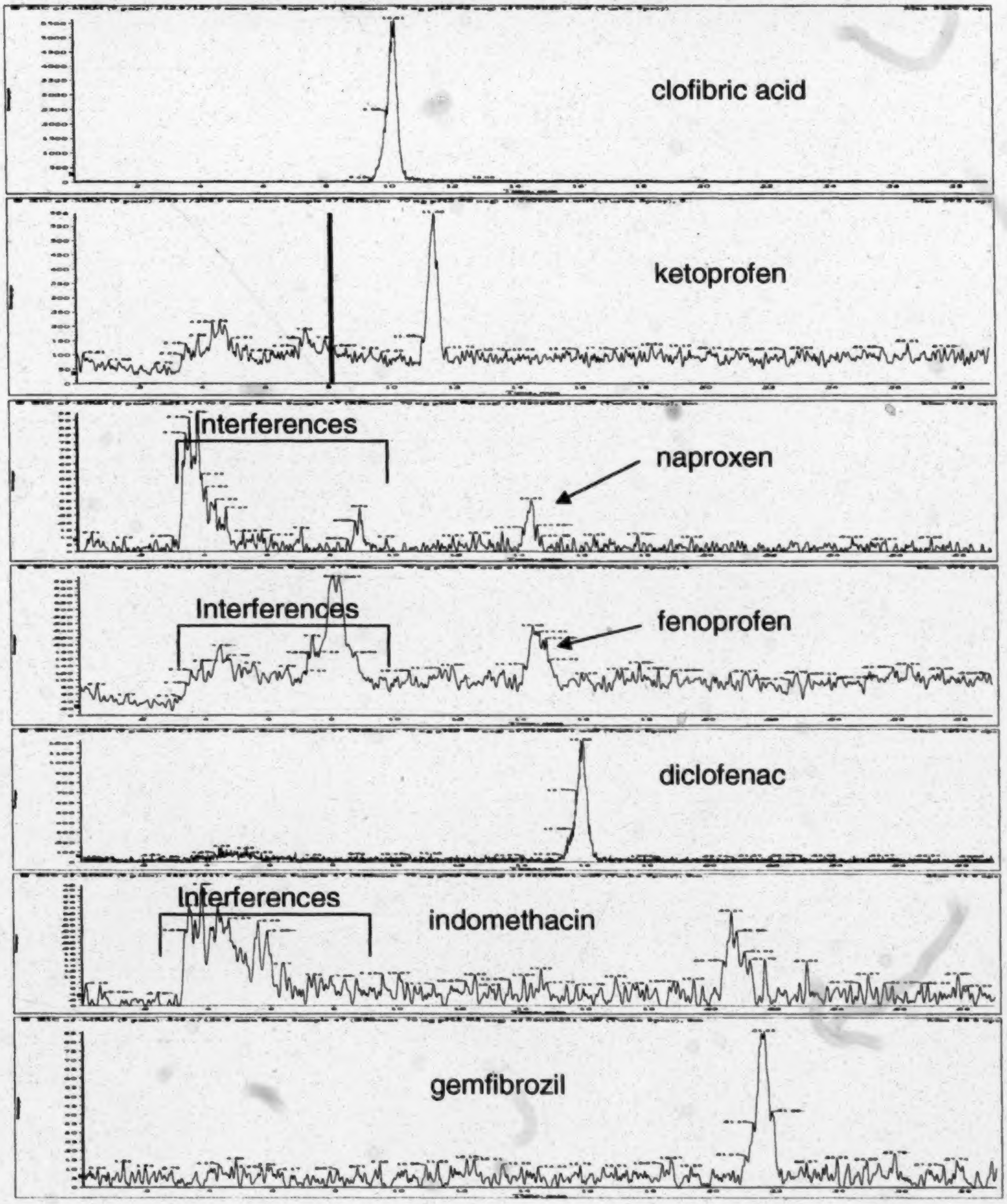


Figure 3.9 Chromatograms of 50 ng/mL mixture of negative mode target analytes extracted from WWTP Effluent at pH 7 on Chiralpak AD-RH

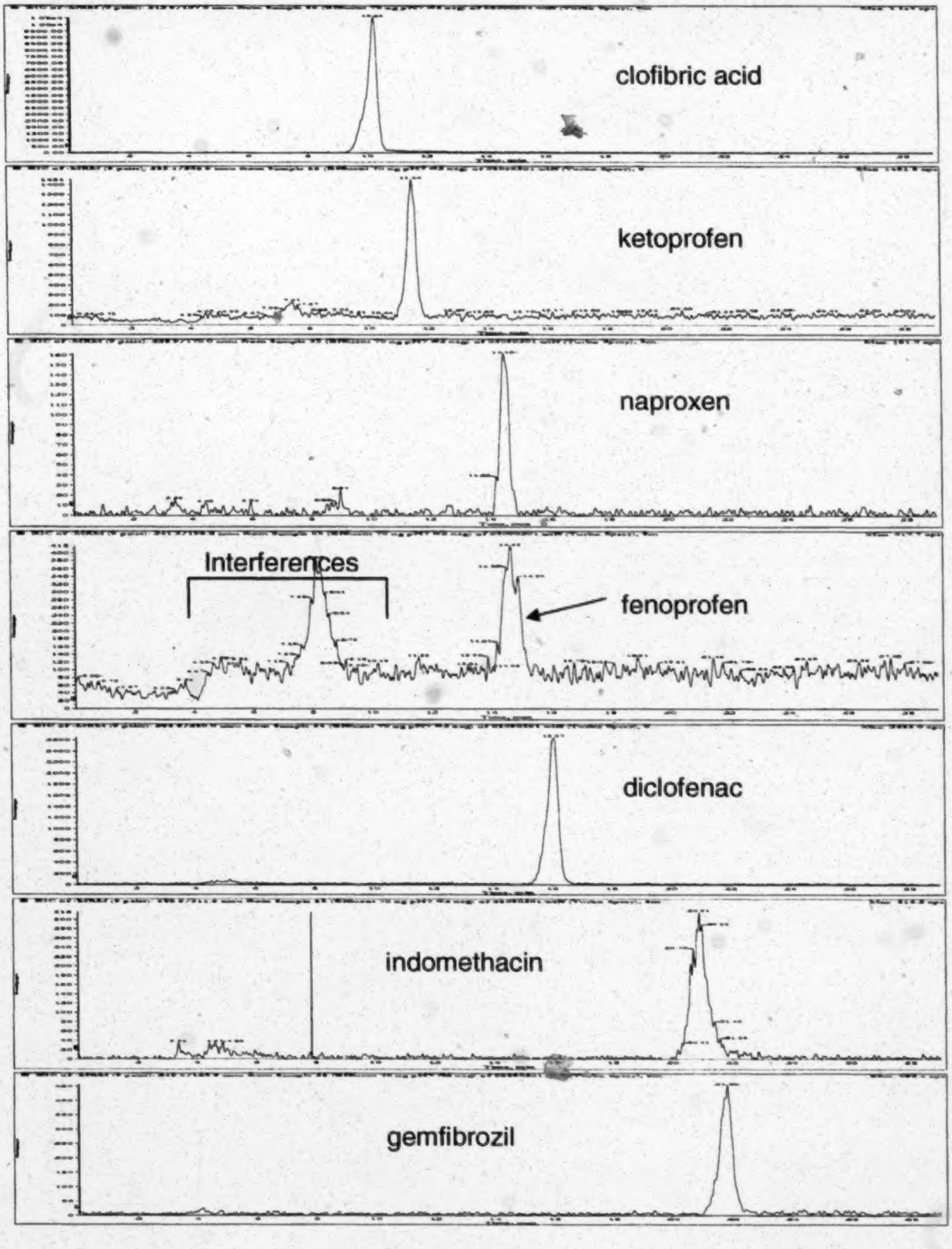


Figure 3.10 Chromatograms of 50 ng/mL mixture of positive mode β-Blockers extracted from WWTP Effluent at pH 2 on Chirobiotic V

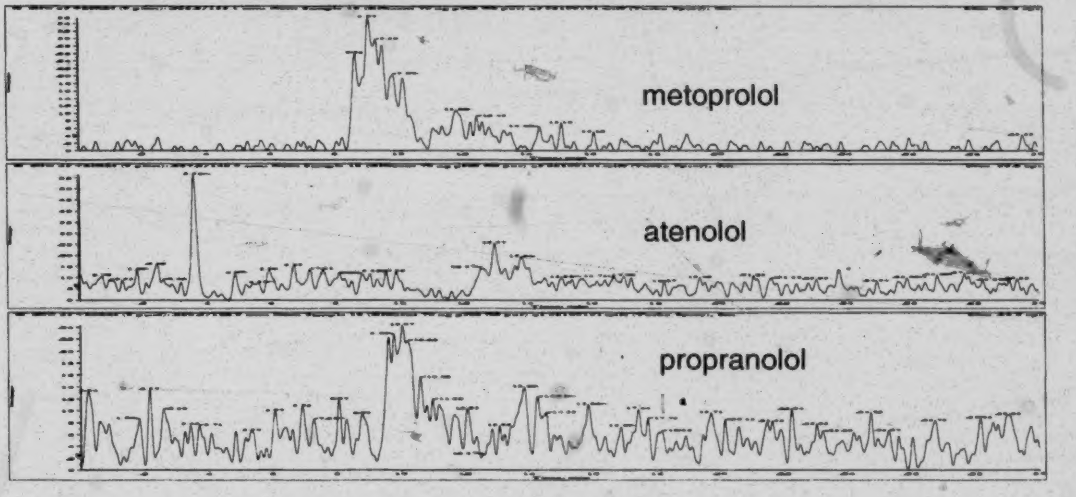
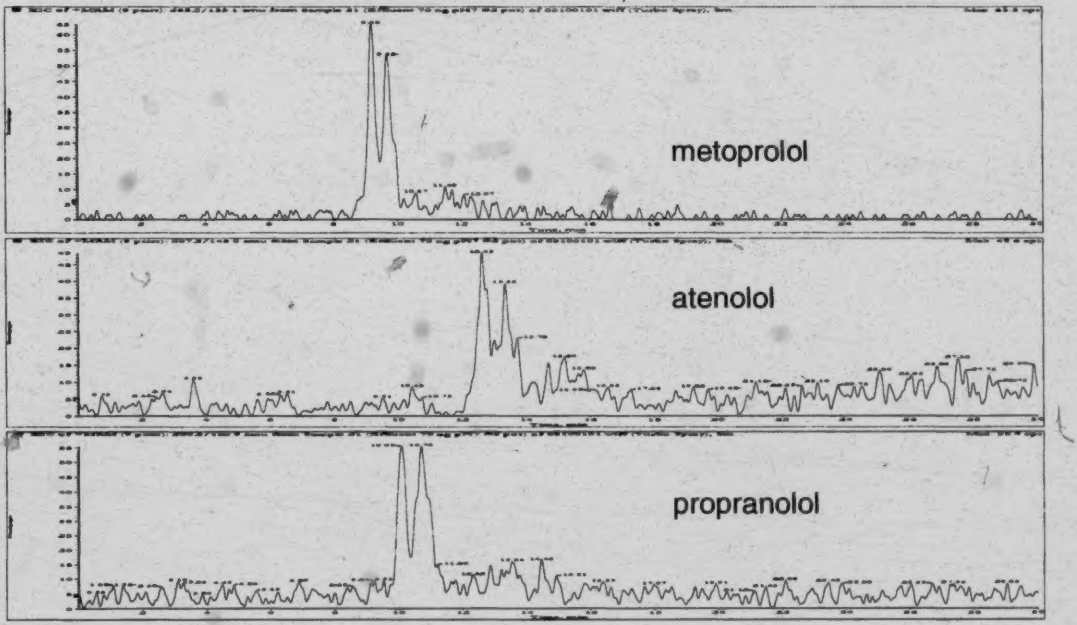


Figure 3.11 Chromatograms of 50 ng/mL mixture of positive mode β-Blockers extracted from WWTP Effluent at pH 7 on Chirobiotic V



The effect of cartridge type on percent recovery was briefly investigated by comparing Waters Oasis HLB solid phase extraction cartridges with Alltech Prevail extract-clean columns. Both cartridges were run under identical conditions. Samples were acidified and filtered before extraction and analytes were eluted using 3 mL of HPLC grade methanol. Most analytes showed much higher percent recoveries on the Oasis cartridges, and for none of the analytes were the Prevail cartridges better (Table 3.9). Using the t-test values and the criteria of $P < 0.05$, only fenoprofen, diclofenac, indomethacin and gemfibrozil are not statistically different. Fluoxetine showed 39% recovery using Oasis versus 1% recovery using Prevail. Caffeine showed 104% and 28% for Oasis and Prevail, respectively. Clofibric acid was not recovered using Prevail, but was recovered at 89% with Oasis. However, two acidic drugs did show reasonable recoveries using Prevail. Indomethacin was recovered at ~73% for both the Oasis and Prevail and gemfibrozil was recovered at 94% and 89% for Oasis and Prevail, respectively. Based on these results, it was decided to continue using only the Oasis cartridges for sample extraction.

The volume of methanol used during the extraction was then considered because too small of an elution volume may result in incomplete elution from the cartridge. Percent recoveries were compared for 3 mL and 6 mL elution volumes (Table 3.10). In nanopure water, all analytes had $P>0.05$ for the two elution volumes and thus were not statistically different. In a river water matrix, only caffeine showed a $P < 0.05$ and was not statistically different between a 3 mL and 6 mL elution volume.

Table 3.9 Percent Recovery using Oasis HLB vs. Prevail C_{18} SPE Cartridges

σ is the standard deviation and N = 3 replicates

Samples were prepared in nanopure water and spiked at 50 ng/mL

β-blockers were not being investigated at the time of this experiment

Drug	Oasis HLB (± σ)	Prevail C_{18} (± σ)	T-test Values
Fluoxetine	39 ± 4	1 ± 0.05	0.009
Caffeine	104 ± 18.0	28 ± 5	0.031
Metoprolol	-	-	
Atenolol	-	-	
Propranolol	-	-	
Clofibric acid	90 ± 5.0	0	0.002
Ketoprofen	83 ± 5.0	32 ± 3	0.002
Naproxen	108 ± 18.0	30 ± 14	0.023
Fenoprofen 1	60 ± 8	44 ± 4	0.250
Fenoprofen 2	77 ± 7	43 ± 2	0.086
Diclofenac	60 ± 1	52 ± 3	0.084
Indomethacin	73 ± 19	72 ± 8	0.480
Gemfibrozil	94 ± 20	89 ± 7	0.420

Indomethacin has a P value of 0.0507 in river water. Testing additional samples may show a 6 mL elution volume is statistically different from the 3 mL elution volume for this drug. Given the results, 6 mL was chosen for the methanol elution volume. Although these results demonstrate that a 6 mL elution volume is generally not statistically different than a 3 mL elution volume, the final conditions selected for SPE used Waters Oasis HLB sample extraction cartridges with a 6 mL elution volume of methanol. Sample pH was not adjusted and a flow rate of 10 mL/min was used during the extraction procedure.

Table 3.10 SPE Recoveries 3 mL vs. 6 mL Extraction Volumes
N = 2

Drug	Nanopure water			River water		
	3 mL	6 mL	t-test value	3 mL	6 mL	t-test value
Fluoxetine	53 ± 7	47 ± 3	0.423	8 ± 4	46 ± 4	0.132
Caffeine	86 ± 13	62 ± 10	0.124	71 ± 1	75 ± 3	0.001
Metoprolol	-	65 ± 23	-	-	60 ± 2	-
Atenolol	-	63 ± 15	-	-	67 ± 5	-
Propranolol	-	72 ± 23	-	-	56 ± 4	-
Clofibric acid	88 ± 9	95 ± 6	0.189	83 ± 16	79 ± 6	0.477
Ketoprofen	66 ± 19	104 ± 4	0.334	83 ± 16	116 ± 2	0.189
Naproxen	96 ± 44	112 ± 10	0.346	97 ± 33	111 ± 1	0.476
Fenoprofen 1	71 ± 32	149 ± 8	0.346	73 ± 47	148 ± 32	0.117
Fenoprofen 2	27 ± 4	136 ± 7	0.209	34 ± 8	98 ± 4	0.404
Diclofenac	81 ± 9	115 ± 1	0.284	78 ± 12	120 ± 2	0.342
Indomethacin	69 ± 9	87 ± 10	0.153	47 ± 15	103 ± 11	0.0507
Gemfibrozil	105 ± 7	119 ± 4	0.331	146 ± 62	117 ± 3	0.425

3.3.2 Method Validation

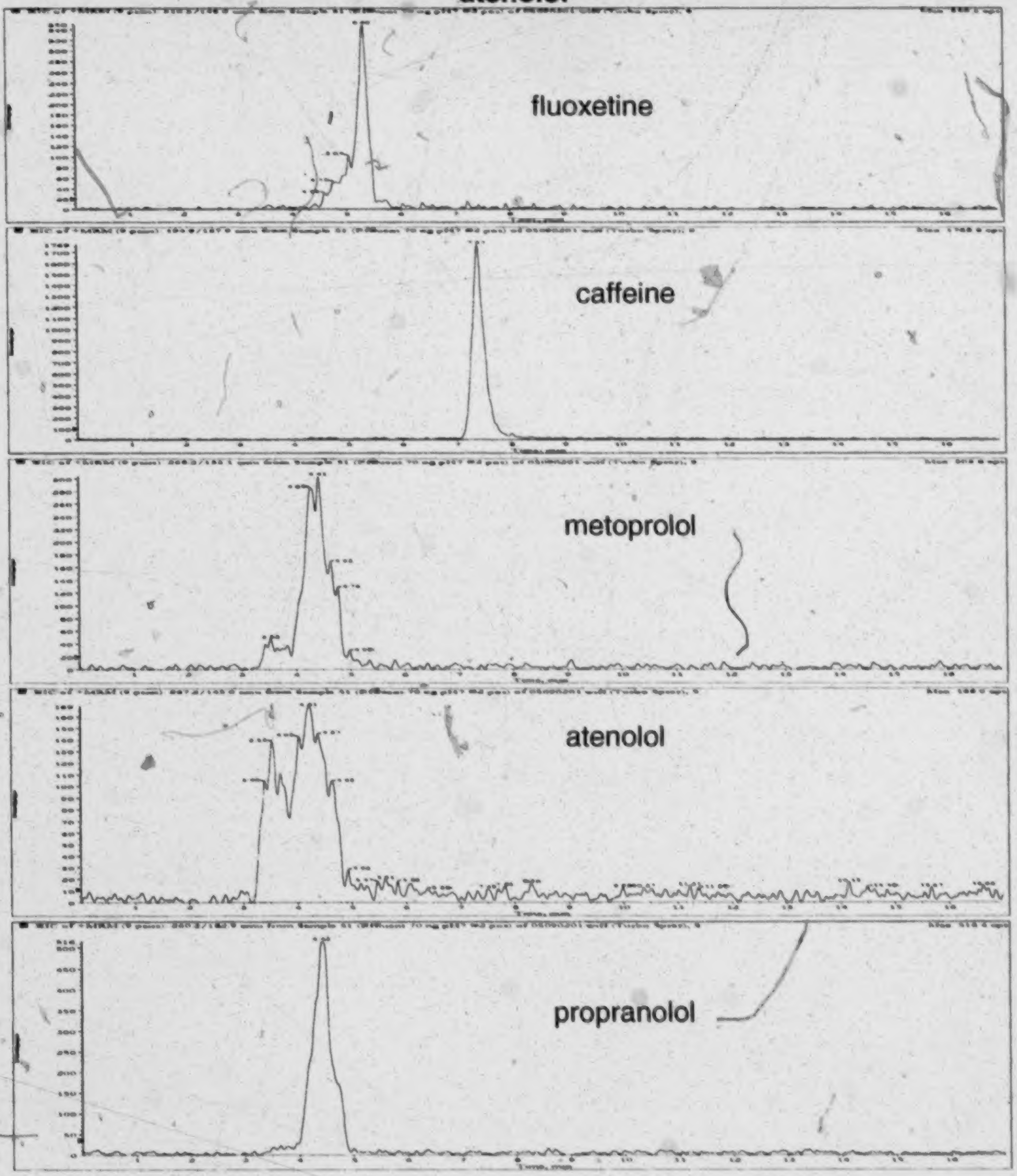
3.3.2.1 Calibration and Linearity

Six calibration curves were prepared between 21 August 2005 and 30 September 2005 over the range 0.1 ng/mL to 100 ng/mL for each analyte by plotting peak area vs. analyte concentration, relative to those of the internal standard. A calibration curve was run each time samples were analyzed. The r^2, slopes and intercepts are shown in Table 3.11. The r^2 on the Chiralpak AD-RH were greater than 0.994 for all but fenoprofen, gemfibrozil and metoprolol, which ranged from 0.9775 to 0.9888. The r^2 of the enantiomers of fenoprofen were not statistically different. This indicates good linearity of the calibration curves and the chromatography on this column has proven to be very reproducible. A racemic mixture is expected to show equal responses to each enantiomer and therefore it is important for the linearity and slopes (relative response factors, or RRFs) for each enantiomer to agree within statistical error. Analytical response on the Whelk-O 1 column was not as linear as the Chiralpak AD-RH, with r^2 ranging from 0.9118 to 0.9863. This was likely due to the less reproducible chromatography seen on the Whelk-O 1. Because the chiral chromatography had degraded on this column over time, it was not used in the analysis of environmental samples. Perhaps the Whelk-O 1 stationary phase needs to be regenerated and would lead to reproducible chiral separation. The Chirobiotic V column had r^2 ranging from 0.9739 to 0.9930 and the enantiomers agree within error. The slopes of the calibration curves correspond to the relative response factors (RRF) of the analytes relative to the internal standards. On the Chiralpak AD-RH column, the fenoprofen enantiomers have RRFs that were not statistically different. The other negative mode target RRFs ranged from 0.0162 (naproxen) to 1.432 (clofibric acid). With the exception of clofibric acid, the negative mode target RRFs had standard deviations less than ± 0.061. The RRF %RSD ranged from 14.2% (clofibric acid) to 34.1% (fenoprofen). The positive mode targets' RRFs ranged from 0.169 (fluoxetine) to 0.295 (propranolol). The standard deviations of the RRFs for the positive mode targets were higher (greater than ± 0.12) than was seen for the negative targets and the %RSD ranged from 60.2% (atenolol) to 76.7% (metoprolol).

Table 3.11 Linearity and RRF of calibration curves for target analytes.
N = 6 and σ is the standard deviation
Note that the merged cells represent the sum of both unresolved stereoisomers

Drug	Chiralpak AD-RH				Chirobiotic V				Whelk-O 1			
	Slope (RRF ± σ)	Slope %RSD	Intercept (± σ)	R^2 (± σ)	Slope (RRF ± σ)	Slope %RSD	Intercept (± σ)	R^2 (± σ)	Slope (RRF ± σ)	Slope %RSD	Intercept (± σ)	R^2 (± σ)
Fluoxetine	0.169 ± 0.126	74.6	0.0046 ± 0.0059	0.9941 ± 0.0041	-	-	-	-	-	-	-	-
Caffeine	0.259 ± 0.157	60.8	0.0028 ± 0.0036	0.9950 ± 0.0027	-	-	-	-	0.224 ± 0.046	20.5	0.006 ± 0.004	0.9297 ± 0.0060
Metoprolol 1	0.181 ± 0.139	76.7	0.0001 ± 0.0044	0.9888 ± 0.0199	0.416 ± 0.0718	17.3	-0.0008 ± 0.0105	0.9878 ± 0.0091	-	-	-	-
Metoprolol 2					0.424 ± 0.1055	24.9	-0.0026 ± 0.0101	0.9757 ± 0.0199	-	-	-	-
S-(-)-Atenolol	0.203 ± 0.122	60.2	0.0058 ± 0.0085	0.9969 ± 0.0015	0.410 ± 0.0914	22.3	-0.0008 ± 0.0147	0.9921 ± 0.0024	-	-	-	-
R-(+)-Atenolol					0.442 ± 0.0848	19.2	0.0017 ± 0.0130	0.9897 ± 0.0045	-	-	-	-
S-(-)-Propranolol	0.295 ± 0.199	67.3	0.0034 ± 0.0035	0.9953 ± 0.0016	0.562 ± 0.0503	8.9	-0.0051 ± 0.0055	0.9739 ± 0.0162	-	-	-	-
R-(+)-Propranolol					0.567 ± 0.1540	27.1	-0.0080 ± 0.0119	0.9930 ± 0.0099	-	-	-	-
Clofibric acid	1.432 ± 0.203	14.2	0.0148 ± .0136	0.9959 ± 0.0018	-	-	-	-	1.166 ± 0.306	26.3	0.0059 ± 0.0063	0.9778 ± 0.0164
Ketoprofen	0.172 ± 0.032	18.6	0.0052 ± 0.006	0.9945 ± 0.0056	-	-	-	-	0.228 ± 0.063	27.7	0.0046 ± 0.0039	0.9636 ± 0.0291
Naproxen	0.0162 ± 0.005	28.8	0.0003 ± 0.0002	0.9948 ± 0.0026	-	-	-	-	0.0856 ± 0.029	33.9	-0.000028 ± 0.000015	0.9827 ± 0.0152
Fenoprofen 1	0.0525 ± 0.018	34.1	0.0009 ± 0.0023	0.9775 ± 0.0161	-	-	-	-	0.230 ± 0.080	34.6	0.0040 ± 0.0020	0.9118 ± 0.0717
Fenoprofen 2	0.0385 ± 0.013	32.7	0.0015 ± 0.0013	0.9801 ± 0.0129	-	-	-	-				
Diclofenac	0.329 ± 0.061	18.6	0.0043 ± 0.0033	0.9967 ± 0.0018	-	-	-	-	0.292 ± 0.048	16.3	-0.0001 ± 0.0001	0.9863 ± 0.0166
Indomethacin	0.0887 ± 0.025	27.7	0.0005 ± 0.0014	0.9942 ± 0.0022	-	-	-	-	0.218 ± 0.119	54.5	-0.0001 ± 0.0001	0.9801 ± 0.0221
Gemfibrozil	0.0369 ± 0.008	20.5	0.0013 ± 0.0020	0.9799 ± 0.0039	-	-	-	-	0.0213 ± 0.006	25.8	-0.000016 ± 0.000006	0.9381 ± 0.0203

Figure 3.12 Chromatograms of positive mode targets in WWTP influents at pH 7 on Chiralpak AD-RH demonstrating possible chiral resolution of atenolol



Fluoxetine, caffeine and clofibric acid were generally tall and wide peaks and the β-blockers were generally low and wide. These non-ideal peak shapes may account for the high standard deviations. At certain times, it appeared as though the β-blockers were almost resolving on the Chiralpak AD-RH. This was especially true of atenolol and is illustrated in Figure 3.12, which shows the separation of the positive mode targets at pH 7 in WWTP influents from Gold Bar. The separation at atenolol did not appear in every chromatogram and was not reproducible enough to conclusively say it had resolved. Perhaps changing the chromatographic conditions or collecting more data points to characterize the peaks would cause reproducible resolution of the peaks. However, since the Chirobiotic V is known to resolve these β-blockers, it was decided to use that column for their separation and abandon further efforts on enantiomer separation on the Chiralpak AD-RH (D'Orazio et al. 2005). The β-blockers' RRFs on the Chirobiotic V seemed quite different from on the Chiralpak AD-RH and ranged from 0.410 to 0.567. This is not surprising because the analytes may not have the same response on each column due to the different chromatographic conditions used. The peak shapes were much better on the Chirobiotic V although the peaks still were somewhat wide (Fig. 3.10). The Whelk-O 1 had RRFs ranging from 0.0213 (gemfibrozil) to 1.166 (clofibric acid) for the negative mode targets. Due to the chromatography problems, the only positive mode target that could be quantified was caffeine and had an RRF of 0.224. With the exception of naproxen, fenoprofen and gemfibrozil, the Whelk-O 1 RRFs agreed within error to those on the Chiralpak AD-RH.

The final factor examined was the intercept of the linear equations. This factor should be zero for all analytes. On the Chiralpak AD-RH, only naproxen, diclofenac and the second enantiomer of fenoprofen are not equal to zero, within error. The fenoprofen intercepts are equal to each other within error, however. All of the enantiomers of the β-blockers have intercepts within error of zero on the Chirobiotic V. The Whelk-O 1 is within error of zero for all analytes tested except naproxen, fenoprofen and gemfibrozil.

The above analysis of slope (RRF), intercept and r^2 for all analytes on the Chiralpak AD-RH, Whelk-O 1 and Chirobiotic V shows that the chromatography of these compounds is both linear and reproducible.

3.3.2.2 Limits of Detection, Limits of Quantification and Percent Recoveries

The limits of detection (LOD = S/N of 3), limits of quantification (LOQ = S/N of 10) and percent recoveries were determined on the QTrap for each column and are found in Tables 3.12 to 3.14. The LOD and LOQ were determined as pg injected from a 25 µL injection (Table 3.12). The Chiralpak AD-RH had LOD ranging from 1.12 pg (caffeine) to 131 pg (gemfibrozil) for standards prepared in methanol. With the exception of ketoprofen, indomethacin and gemfibrozil, all of the LOD are less than 20 pg on this column. The LOQ ranged from 0.75 pg (clofibric acid) to 191 pg (ketoprofen), with the exception of gemfibrozil at 437 pg. The Whelk-O 1 had LOD between 0.16 pg (caffeine) and 547 pg (gemfibrozil). The LOQ ranged from 0.54 pg (caffeine) to 1820 pg (gemfibrozil). Gemfibrozil showed quite high LOD and LOQ on this column compared to those on the Chiralpak AD-RH.

Table 3.12 Instrumental Limits of Detection and Quantification

Results are presented as pg injected for a 25 μL injection volume

Drug	Chiralpak AD-RH		Whelk-O 1		Chirobiotic V		WWTP Influent		WWTP Effluent	
	LOD (pg)	LOQ (pg)	LOD (pg)	LOQ (pg)	LOD (pg)	LOQ (pg)	LOD (pg)	LOQ (pg)	LOD (pg)	LOQ (pg)
Fluoxetine	3.38	11.3	-	-	-	-	91.2	304	155	517
Caffeine	1.12	3.73	0.16	0.54	-	-	70.4	235	98.9	330
Metoprolol	10.6	35.4	-	-	36.1	120	104	348	217	723
Atenolol	3.26	10.9	-	-	8.66	8.66	272	907	15.6	51.8
Propranolol	3.15	10.5	-	-	27.8	27.8	42.6	142	54.7	182
Clofibric acid	0.75	2.50	18.6	62.0	-	-	7.07	23.6	0.81	2.71
Ketoprofen	57.3	191	207	691	-	-	221	736	59.4	198
Naproxen	17.3	57.7	14.1	46.9	-	-	955	3180	475	1580
Fenoprofen	12.4	41.4	246	820	-	-	2030	6760	1480	4930
Diclofenac	19.7	65.6	16.0	53.3	-	-	120	398	110	366
Indomethacin	39.3	131	21.2	70.6	-	-	403	1340	371	1240
Gemfibrozil	131	437	547	1820	-	-	914	3050	395	1320

The Chirobiotic V had LOD ranging from 8.66 pg (atenolol) to 36.1 pg (metoprolol). The LOQ ranged from 28.9 pg (atenolol) to 120 pg (metoprolol). All three β-blockers had higher LOD and LOQ on the Chirobiotic V. Caffeine, naproxen, diclofenac and indomethacin all had lower LOD and LOQ on the Whelk-O 1 compared to the Chiralpak AD-RH. They were not compared to the Chirobiotic V because they could not be quantified. Limits of detection and quantification were determined in WWTP influents and effluents using the Chiralpak AD-RH (Table 3.12). The influent LOD ranged from 7.07 pg (clofibric acid) to 2030 pg (fenoprofen) and the LOQ ranged from 23.6 pg (clofibric acid) to 6760 pg (fenoprofen). The effluent LOD ranged from 0.81 pg (clofibric acid) to 1480 pg (fenoprofen) and the LOQ ranged from 2.71 pg (clofibric acid) to 4930 pg (fenoprofen). Interestingly, the positive mode targets generally showed lower LOD and LOQ in influent samples, with the exception of atenolol. This was unexpected because the influent is a more complex matrix than is the effluent and is expected to have higher LOD and LOQ. The negative mode targets all had lower LOD and LOQ in the effluent samples, as was expected. Typical LOD found in literature (Table 3.13) range from 5 ng/L sample to 20 ng/L sample (Cahill et al. 2004; Metcalfe et al. 2003b; Miao et al. 2002). The values reported in this thesis are comparable when expressed as ng/L environmental sample (Table 3.13). The WWTP influent samples were from 100 mL samples while the WWTP effluent samples were from 500 mL samples.

Table 3.13 Comparison of LOD (ng/L) in Environmental Samples to Typical Literature LOD (ng/L)

WWTP influent sample volume = 100 mL
WWTP effluent sample volume = 500 mL

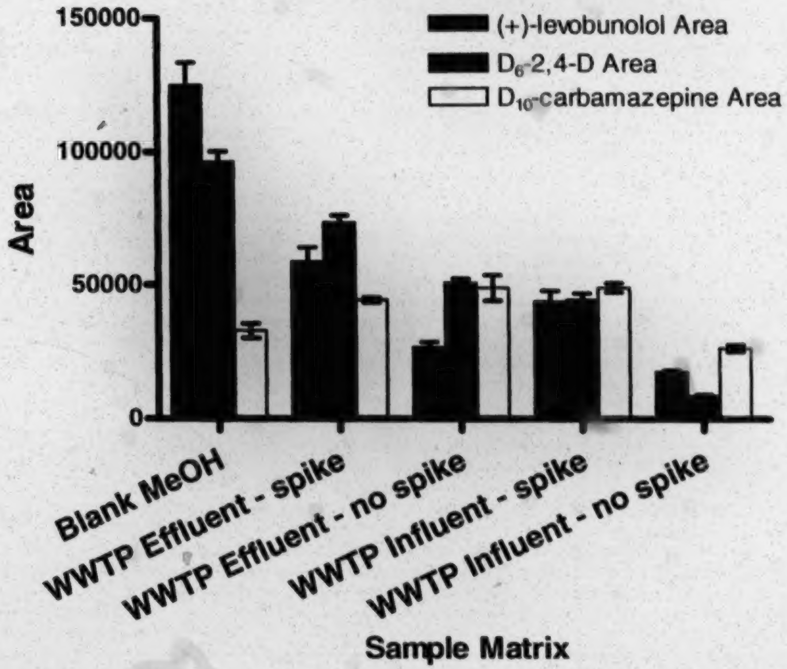
Drug	Literature Values LOD (ng/L)	WWTP Influent LOD (ng/L)	WWTP Effluent LOD (ng/L)
Fluoxetine	18 [a]	36.5	12.4
Caffeine	14 [a]	28.2	7.91
Metoprolol	-	41.7	17.4
Atenolol	-	109	1.24
Propranolol	-	17.0	4.37
Clofibric acid	5-10 [b,c]	2.83	0.06
Ketoprofen	5-20 [b,c]	88.3	4.75
Naproxen	5-10 [b,c]	382	38.0
Fenoprofen	5-10 [b,c]	811	118
Diclofenac	5-10 [b,c]	47.8	8.79
Indomethacin	10 [c]	161	29.7
Gemfibrozil	5 [b]	366	31.6

a = (Cahill et al. 2004), b = (Metcalfe et al. 2003b), c = (Miao et al. 2002)

Percent recoveries were calculated using the Chiralpak AD-RH for standards, WWTP effluents and WWTP influents (Table 3.6). The standards were spiked at a level of 70 ng/mL while the WWTP samples were spiked at 50 ng/mL. At the experimental conditions of pH 7, fluoxetine and caffeine had the lowest recoveries among the nanopure water standards (49% and 65%, respectively). The remaining pharmaceuticals had recoveries > 71%. In the WWTP effluent samples, metoprolol and clofibric acid showed poorer recoveries, at 59%. Except for propranolol, the remaining analytes had percent recoveries

>100% in effluent samples. For WWTP influent samples, all but atenolol and diclofenac had percent recoveries > 100%. Caffeine and gemfibrozil in particular had very high recoveries. The high recoveries are likely due to internal standards that are not ideal for the analytes or to a spiked concentration that is too low. Although not used in this thesis, absolute recoveries calculated without the use of internal standards could be used to determine if the internal standards were causing the high recoveries. If the internal standard and analyte are not structurally similar, they may not be affected to the same extent by ion suppression. If the internal standard is suppressed more than the analyte or if the recovery is different, the analyte concentration will be erroneously high. Figure 3.13 illustrates the ion suppression and enhancement seen for the internal

Figure 3.13 Ion Suppression and Enhancement of Internal Standards



standards. The areas of (+)-levobunolol and D_6-2,4-D are maximal in blank methanol and generally decrease as matrix complexity increases while D_{10}-carbamazepine shows highest area in the WWTP effluent and influent samples. These results indicate (+)-levobunolol and D_6-2,4-D are suppressed while D_{10}-carbamazepine is enhanced as a result of the matrix. For best results, standard addition should be used instead of internal standards in these cases. The matrix effects would be removed because there is no difference between the standard and sample matrix. The downside to standard addition is that the samples can only be used once, since the standards are being added to the sample and sample extraction can be a long process. The retention time of the internal standard relative to that of the analyte may also play a role because co-eluting matrix material will elute at different times. If the internal standard and analytes do not elute close to each other, they may experience ion suppression or enhancement to different extents. This is why the ideal internal standard chosen for an analysis would be an isotopically labelled version of the target analyte. A related issue is the differential ion suppression that may result from the different retention times of the two enantiomers. If the separation is sufficiently large, the two enantiomers may be affected by different matrix compositions. If this happens, a racemic mixture may appear non-racemic and incorrect conclusions may be drawn regarding fate processes. Different internal standards could be used for each of the enantiomers but would make quantitative comparisons between the two enantiomers difficult because they are not quantified relative to the same internal standard. A labelled version of the chiral analyte could be used as an internal standard. Depending on the resolution between the enantiomers, this may not be the best choice due to difficulties in reproducibly integrating non-baseline resolved peaks. It also would make quantitative comparisons of the enantiomers difficult, as mentioned above. The best choice would be an internal standard that elutes between the two peaks and is a compromise to both. This is not expected to be a problem in this work, however, as the chiral resolutions presented here are not baseline or better. Overall, the recoveries presented above are good but there are noticeable differences in recoveries between the matrices that are likely related to different ionization efficiencies between the analytes and the internal standards.

The above results show the methods used for the determination of chiral pharmaceuticals in environmental water samples are sensitive and selective and the chiral columns investigated are stable enough for routine analysis and quantification.

3.3.2.3 Precision and Accuracy

The LC-MS/MS instrumentation must provide precise and accurate results both within the same day and between days to be considered analytically sound. Intra- and inter-day precision was investigated by repeatedly analyzing a 100 ng/mL standard solution and observing the short-term and long-term RSDs of the measured concentration under identical conditions. Table 3.14 presents precision and accuracy data for both the Chiralpak AD-RH and the Chirobiotic V. The Chiralpak AD-RH inter-day precision was measured by comparing the measured concentration for a known 100 ng/mL sample on twelve days between 11 May 05 and 16 September 2005 and calculating the %RSD. The Chirobiotic V inter-day precision was calculated from three samples between 2 August 2005 and 19 September 2005. The Chiralpak AD-RH inter-day precision ranged from 1.7 to 12.2% while the Chirobiotic V inter-day precision ranged from 4.2 to 11.3%. The lowest precision values for each column, 12.2% and 11.3%, were for fenoprofen and atenolol, respectively. These values are likely due to chiral separation that results in low, wide peaks that are not completely resolved. The intra-day precision was calculated by running five samples at 100 ng/mL on 30 September 2005. The intra-day precision on the Chiralpak AD-RH ranged from 0.9% (diclofenac) to 6.1% (gemfibrozil), with the exception of fenoprofen. Fenoprofen was higher, with 14.6% and 12.8% precision for the two enantiomers. Again, this is likely due to the chiral separation. With the exceptions noted above, all precisions were less than 10%, which shows these chiral columns are capable of sustained, long-term use with little to no degradation of chromatography. The values used for accuracy were the concentrations calculated using the calibration curves, compared to the nominal concentration of those standards, expressed as a percentage. All standards were prepared in methanol and were replaced within three months. Accuracy values ranged from 93.9% to 115.8% on the Chiralpak AD-RH and 97.5% to 107% on the Chirobiotic V. Gemfibrozil is slightly high, at 115.8%, but the remaining drugs are 100 ± 7% on both the Chiralpak AD-RH and Chirobiotic V. This shows excellent accuracy over time and confirms a three-month period between standards preparation is acceptable.

Table 3.14 Precision and Accuracy

Drug	Chiralpak AD-RH			Chirobiotic V	
	Accuracy (%)	Precision (%RSD) Intra-day	Inter-day	Accuracy (%)	Precision Inter-day
Fluoxetine	101.0	1.6	7.8	-	-
Caffeine	100.	2.3	5.3	-	-
Metoprolol 1	99.3	2.1	3.2	97.5	8.2
Metoprolol 2	-	-	-	107.0	5.5
S-(-)-Atenolol	101.9	3.0	1.9	99.1	8.7
R-(+)-Atenolol	-	-	-	100.4	11.3
S-(-)-Propranolol	100.8	3.4	5.9	104.7	11.2
R-(+)-Propranolol	-	-	-	101.2	4.2
Clofibric acid	100.5	2.6	1.7	-	-
Ketoprofen	106.9	1.0	2.3	-	-
Naproxen	101.8	2.0	5.6	-	-
Fenoprofen 1	93.9	14.6	12.2	-	-
Fenoprofen 2	102.3	12.8	8.4	-	-
Diclofenac	106.5	0.9	2.7	-	-
Indomethacin	106.6	1.5	3.4	-	-
Gemfibrozil	115.8	6.1	9.6	-	-

Chiral separation was seen for metoprolol, atenolol and propranolol only on the Chirobiotic V and fenoprofen separation was seen only on the Chiralpak AD-RH. The sum of single enantiomer concentrations for the β-blockers were compared to the total concentration of the unresolved compound to compare the inter-column results (Table 3.15). Fenoprofen could not be compared because it was not seen on the Chirobiotic V. The standard concentration used for comparison was 100 ng/mL. Metoprolol standards show a sum concentration of 102.4 ± 5.9 ng/mL and an unresolved concentration of 100.4 ± 3.3 ng/mL.

Table 3.15 Comparison of Resolved Enantiomer Total Concentration and Unresolved Enantiomer Concentration

Note: bold font indicates which numbers are significantly different

Standards

Drug	Resolved Standards (ng/mL)	Unresolved Standards (ng/mL)
Metoprolol	102 ± 6	100 ± 3
Atenolol	100 ± 10	100 ± 2
Propranolol	103 ± 6	101 ± 6
Fenoprofen	100 ± 5	-

Influents

Drug	Edmonton WWTP #1		Edmonton WWTP #2		LLB WWTP	
	Resolved (ng/L)	Unresolved (ng/L)	Resolved (ng/L)	Unresolved (ng/L)	Resolved (ng/L)	Unresolved (ng/L)
Metoprolol	522 ± 27	658 ± 15	622 ± 67	661 ± 42	**346 ± 33**	**773 ± 69**
Atenolol	689 ± 51	471 ± 35	**2390 ± 56**	**1720 ± 78**	790 ± 213	620 ± 51
Propranolol	92 ± 7	68 ± 8	55 ± 40	77 ± 15	46 ± 3	395 ± 387
Fenoprofen	40 ± 16		152 ± 82		ND	

Effluents

Drug	Edmonton WWTP #1		Edmonton WWTP #2		LLB WWTP	
	Resolved (ng/L)	Unresolved (ng/L)	Resolved (ng/L)	Unresolved (ng/L)	Resolved (ng/L)	Unresolved (ng/L)
Metoprolol	**410 ± 17**	**525 ± 17**	519 ± 30	661 ± 42	**346 ± 33**	**773 ± 69**
Atenolol	776 ± 49	878 ± 58	899 ± 57	1720 ± 78	790 ± 213	620 ± 51
Propranolol	**83 ± 3**	**103 ± 11**	**85 ± 8**	**66 ± 4**	46 ± 3	395 ± 387
Fenoprofen	334 ± 69		928 ± 137		0	

Atenolol standards show a summed concentration of 99.7 ± 10.0 ng/mL and an unresolved concentration of 100.2 ± 1.9 ng/mL while propranolol standards had a summed concentration of 102.9 ± 6.3 ng/mL and an unresolved concentration of 101.0 ± 6.0 ng/mL. Thus the concentration results between columns agree within standard deviation for the standards. This comparison was also made for WWTP influents and effluents (Table 3.15). The agreement is not as close as was found for the standards and is not constant among analytes. Only atenolol showed agreement between resolved and unresolved concentrations for all effluent samples. For the first set of effluent samples taken from Gold Bar, only atenolol shows agreement within error of resolved and unresolved sums.

In the influent samples, none of the drugs show agreement. In the second set of effluent samples from Edmonton, both metoprolol and atenolol agree within error. The influent samples show agreement between metoprolol and propranolol. In the Lac la Biche effluents and influents, atenolol and propranolol agreed within error. This lack of agreement is likely related to the chromatography since none of the β-blockers were baseline resolved and slight errors may have arisen during the quantification process. If further samples were quantified, the results would likely improve.

3.3.2.4 Analyte Concentrations in Wastewater and Surface Waters

The methods were applied to a number of sample matrices, including surface waters and WWTP influents and effluents in Edmonton, Alberta, Canada and Lac la Biche, Alberta, Canada. Acidic and basic pharmaceuticals were investigated. The Edmonton WWTP treats 310 ML/day and serves a population of over 712,000 people in the greater Edmonton area. It consists of primary treatment, secondary biological treatment and tertiary UV treatment. Influent samples were taken from raw influent before the grit tanks and the effluent samples were taken after the UV treatment. The Lac la Biche WWTP treats 1.8 ML/day and serves a population of 3000 people. It consists of primary treatment and aeration. Influent samples were taken on 20 September 2005 from the raw influent and the effluent samples were taken after the primary treatment aeration.

Table 3.16 presents the concentrations found in both the Edmonton and Lac la Biche WWTP and Field Lake surface waters. Edmonton WWTP effluents had concentrations of pharmaceuticals ranging from 0.1 ng/L (clofibric acid) to 1.97 µg/mL (diclofenac) while the influent concentrations ranged from 0.73 ng/L (clofibric acid) to 202 µg/L (caffeine). The concentrations and standard deviations between the two sets of Edmonton WWTP samples were compared to see if the concentrations were different over time. The values were deemed to be the same if they agreed within standard deviation. Fluoxetine, propranolol, ketoprofen, naproxen and gemfibrozil were present in both the first and second Edmonton WWTP effluent samples at the same concentrations. Caffeine, metoprolol, atenolol, clofibric acid, fenoprofen and indomethacin had different concentrations for the two Edmonton WWTP effluent samples. In the influent samples, propranolol, clofibric acid, ketoprofen, fenoprofen and diclofenac had the same concentrations in both the first and second Edmonton WWTP samples.

Table 3.16 Concentrations and enantiomer fractions (EFs) of Pharmaceuticals in Edmonton and Lac la Biche WWTP Influent and Effluent and in Surface Waters of Field Lake

Drug	Edmonton WWTP #1 (30/08/05)				Edmonton WWTP #2 (13/09/05)				Lac La Biche WWTP (20/09/05)				Field Lake (20/09/05)	
	Influent		Effluent		Influent		Effluent		Influent		Effluent			
	Ave Conc (ng/L)	EF	Ave Conc (ng/L)	EF	Ave Conc (ng/L)	EF	Ave Conc (ng/L)	EF	Ave Conc (ng/L)	EF	Ave Conc (ng/L)	EF	Ave Conc (ng/L)	EF
Fluoxetine	8.0 ± 4.2	n/a	41.9 ± 7.3^{a}		18.8 ± 2.6^{b}		40.9 ± 9.7^{a}		n.d.		6.3 ± 3.0^{b}		n.d	
Caffeine	33300 ± 5390		123 ± 6		201000 ± 5510		34.1 ± 13.8^{a}		321000.0 ± 16900		4330 ± 323		301 ± 96	
Metoprolol 1	283 ± 22	0.408 ± 0.038	209 ± 11	0.519 ± 0.020	291 ± 40	0.430 ± 0.066	249 ± 20	0.495 ± 0.013	176.0 ± 7.1	0.599 ± 0.005	87.4^{c}	0.524 ± 0.039	n.d	n/a
Metoprolol 2	239.3 ± 15.5		202 ± 12		331 ± 54		270 ± 23		170 ± 31		83.4^{c}		n.d	
S-(-)-Atenolol	378.7 ± 48.4^{a}	0.476 ± 0.010	484 ± 37	0.404 ± 0.008	1110 ± 167	0.516 ± 0.015	427 ± 6	0.491 ± 0.009	410 ± 180^{a}	0.415 ± 0.120	100.2^{c}	0.426 ± 0.036	n.d	n/a
R-(+)-Atenolol	310.0 ± 17.3^{a}		292 ± 32		1280 ± 194		473 ± 46		381 ± 114^{a}		64.2^{c}		n.d	
S-(-)-Propranolol	48.1 ± 6.4^{a}	0.466 ± 0.016	45.6 ± 0.4	0.358 ± 0.038	24.5 ± 18.5^{b}	n/a	45.3 ± 7.4	0.491 ± 0.016	23.1 ± 2.2	n/a	10.0^{a,c}	0.374 ± 0.049	n.d	n/a
R-(+)-Propranolol	44.1 ± 2.9^{a}		37.4 ± 3.0		30.1 ± 35.3^{b}		39.9 ± 3.0		22.5 ± 1.6		8.9^{a,c}		n.d	
Clofibric acid	0.7 ± 0.1^{b}		0.4 ± 0.1		2.6 ± 2.3^{b}		0.1 ± 0.1^{b}		n.d.		n.d.		n.d	
Ketoprofen	143.2 ± 36.3^{a}		12.1 ± 6.7^{a}		157 ± 74^{a}		5.6 ± 2.6^{b}		310 ± 0.7		n.d.		n.d	
Naproxen	17900 ± 1250		125 ± 10^{a}		25300 ± 3670		120 ± 51^{a}		10100 ± 205		645 ± 85		22.2 ± 18.9	
Fenoprofen 1	25.0^{b,c}	n/a	187 ± 69	0.253 ± 0.024	74.3 ± 52.2^{a}	n/a	421 ± 87^{a}	0.198 ± 0.015	n.d	n/a	n.d.	n/a	n.d	n/a
Fenoprofen 2	15.2 ± 16.2^{b}		147 ± 3		78.0 ± 63.3^{a}		508 ± 105		n.d.		n.d.		n.d	
Diclofenac	5200.0 ± 423.3		1970 ± 54		5330 ± 871		1980 ± 347		2110 ± 78		221 ± 16		5.8 ± 4.5^{a}	
Indomethacin	339.8 ± 181.4^{a}		116 ± 3		620 ± 65		159 ± 23		1120 ± 28		n.d.		n.d	
Gemfibrozil	4050 ± 261		577 ± 33		3150 ± 532		484 ± 63		5650 ± 149		1080 ± 28		140 ± 19	

^{a}= at or below LOQ ^{b}= at or below LOD ^{c} = number based on single sample

n/a = data was not able to be determined

n.d. = no peak was seen

The concentrations of fluoxetine, caffeine, metoprolol, atenolol, naproxen, indomethacin and gemfibrozil were different for the two influents. Fluoxetine, naproxen and ketoprofen were present below the LOQ in both Edmonton effluents. Caffeine, clofibric acid and fenoprofen were below the LOQ in the second set of Edmonton effluents. Propranolol, clofibric acid, ketoprofen and fenoprofen were below the LOQ in both of the Edmonton influents. Atenolol and indomethacin were below LOQ in the first influent samples while fluoxetine was below LOQ in the second influent samples. The Lac la Biche WWTP had effluent concentrations ranging from 6.3 ng/L to 4.33 µg/L while the influents ranged from 22.5 ng/L to 321 µg/L. Compared to the Edmonton WWTP samples, caffeine, ketoprofen and indomethacin were at higher concentrations in Lac la Biche. Propranolol was below the LOQ in the effluents while atenolol and propranolol were below the LOQ in the influent. The samples from Field Lake, into which the Lac la Biche WWTP sewage lagoons drain, showed only caffeine, naproxen, diclofenac and gemfibrozil. All of their concentrations were much lower than are seen in the Lac la Biche WWTP effluent, which is expected due to dilution as the analytes enter the lake. Diclofenac was below the LOQ. These four analytes had the highest effluent concentrations of the drugs investigated. It is likely the other analytes could be detected in Field Lake if larger sample volumes were extracted, although photolysis and sedimentation could be occurring in this natural environment and may affect the amounts of pharmaceuticals present.

Table 3.17 lists the percent elimination seen during the wastewater treatment process. In the first set of Edmonton WWTP samples, elimination was seen for caffeine, metoprolol, propranolol, clofibric acid, ketoprofen, naproxen, diclofenac, indomethacin and gemfibrozil and ranged from 5% to 100%. Although still present at high concentrations in effluents, caffeine and naproxen were eliminated quantitatively (>99%). Ketoprofen was also efficiently eliminated (92%). Conversely, the β-blockers were not efficiently eliminated (5-26%) and have influent and effluent concentrations that are nearly the same.

Table 3.17 Percent Elimination of Pharmaceuticals between Influent and Effluent

Drug	Edmonton WWTP #1	Edmonton WWTP #2	LLB WWTP
Fluoxetine	n/a	n/a	n/a
Caffeine	100	100	99
Metoprolol 1	26	14	50
Metoprolol 2	16	19	51
S-(-)-Atenolol	n/a	62	76
R-(+)-Atenolol	6	63	83
S-(-)-Propranolol	5	n/a	57
R-(+)-Propranolol	15	n/a	60
Clofibric acid	45	98	n.d.
Ketoprofen	92	96	>99
Naproxen	99	100	94
Fenoprofen 1	n/a	n/a	n.d.
Fenoprofen 2	n/a	n/a	n.d.
Diclofenac	62	63	89
Indomethacin	66	74	>99
Gemfibrozil	86	85	81

n/a = could not be quantified because effluent concentrations were higher than influent concentrations
n.d. = no peak was detected

Unexpectedly, fluoxetine, atenolol and fenoprofen showed higher concentrations in the effluents than the influents. Perhaps there is some process within the plant that concentrates these analytes between the influent and effluent sampling points or an unknown source that introduces raw influent past the point where the influent were sampled. Drugs may also be released from their Phase II metabolites during the wastewater treatment process, resulting in higher concentrations in the effluents.

In the second set of Edmonton WWTP samples, caffeine and naproxen were also eliminated quantitatively (>99%) although clofibric acid and ketoprofen were also efficiently eliminated, at 98% and 96%, respectively. Metoprolol was not efficiently eliminated (14-19%) and fluoxetine, propranolol and fenoprofen had higher effluent than influent concentrations.

The Lac la Biche WWTP showed high elimination of caffeine, ketoprofen and indomethacin (>99%) and efficient elimination of naproxen and diclofenac (>89%). The β-blockers were more efficiently eliminated in Lac la Biche than in the Edmonton WWTP. This was unexpected due to the lower degree of processing in that WWTP compared to the Edmonton WWTP. Perhaps wastewater at the Lac la Biche WWTP has a longer residence time than Edmonton that would result in more time for the analytes to degrade before being released into the environment. Similar to the Edmonton results, fluoxetine was at a higher concentration in the effluents than in the influents. Clofibric acid and fenoprofen were not detected in the Lac la Biche WWTP samples.

These pharmaceuticals have been investigated in WWTP around the world. The specific details have been discussed in Chapter 2.3.3 but some are repeated here for comparison purposes. Table 3.18 presents a comparison of select literature WWTP concentrations and elimination rates in comparison to the values presented in this thesis. Fluoxetine was found at similar concentrations to those found in Ontario WWTP samples by Metcalfe et al. (Metcalfe et al. 2003b). Our caffeine concentrations were found to be much higher (4327 ng/L) than those found by Metcalfe et al. (2003) in Ontario WWTP (667 ng/L). However, the removal rates in Alberta were also higher than the Buerge et al. (2003) values (>99% in Alberta vs. >80%). The metoprolol concentrations in Alberta were lower than those found in German WWTP samples and also had much lower elimination rates (16-51% in Alberta vs. 83% in Germany) (Ternes 1998). Atenolol was found at concentrations much higher than those by Bendz et al. (2005) in Swedish WWTP samples (up to 1276.6 ng/L in Alberta vs. 160 ng/L in Sweden). Ternes et al. (1998) determined much higher concentrations of propranolol in German WWTP effluents than were found in Alberta (up to 2200 ng/L in Germany vs. 45.6 ng/L in Alberta). They also had higher elimination rates than were seen in Alberta (96% in Germany vs. 5-60% in Alberta) (Ternes 1998). Clofibric acid showed much lower concentrations in Alberta WWTP samples than were seen in Ontario WWTP samples, but better elimination rates were seen in Alberta WWTP (Buser et al. 1999; Metcalfe et al. 2003b). Metcalfe et al. (2003) found ketoprofen concentrations at much higher concentrations (up to 5700 ng/L) in Ontario WWTP than were seen in Alberta (up to 309.5 ng/L).

Table 3.18 Literature Comparison of WWTP Concentrations And Elimination Rates

n/a = could not be determined

	Thesis Values		Select Literature Values	
Drug	**Concentrations (ng/L)**	**Removal Rates (%)**	**Concentrations (ng/L)**	**Removal Rates (%)**
Fluoxetine	6.3 – 41.9	n/a	38 – 99 [a]	
Caffeine	34 – 4330	>99	22 – 667 [a]	>80 [b]
Metoprolol	83.4 – 331	16 – 51	Up to 590 [f]	83 [f]
Atenolol	64.2 – 1270	6 – 83	30 – 160 [g]	
Propranolol	8.9 – 45.6	5 – 60	Up to 2200 [f]	96 [f]
Clofibric acid	0.05 – 0.73	45 – 98	1 – 44 [a]	34 – 50 [c]
Ketoprofen	5.6 – 310	92 - >99	Up to 5700 [e]	
Naproxen	120 – 645	>94	12500 – 33900 [a,e]	>90 [a]
Fenoprofen	15.2 – 508	n/a	62 – 405 [a]	
Diclofenac	221 – 5330	62 – 89	990 – 1300 [d,e]	
Indomethacin	116 – 159	64 - >99	10 – 378 [a]	
Gemfibrozil	484 – 1080	85	5 – 484 [a, h]	34 – 50 [a]

a = (Metcalfe et al. 2003b), b = (Buerge et al. 2003), c = (Buser et al. 1999), d = (Tixier et al. 2003), e = (Metcalfe et al. 2003a), f = (Ternes 1998), g = (Bendz et al. 2005), h = (Hernando et al. 2004)

No elimination rates were given for that study, but Alberta WWTP are efficiently removing ketoprofen (92 to >99%). The concentrations of naproxen found in Albertan WWTP samples are much lower than those found by Metcalfe et al. (2003) during a survey of Canadian WWTP. The elimination rates in Alberta are comparable to those in the Canadian WWTP studied by Metcalfe et al. (2003). Fenoprofen showed comparable concentrations to those seen in Ontario WWTP samples (Metcalfe et al. 2003b). Diclofenac had higher concentrations that those found in either Swiss or other Canadian WWTP samples (Metcalfe et al. 2003a; Tixier et al. 2003). Indomethacin was found at comparable levels to those seen in Ontario WWTP and showed 64 to >99% elimination rates. Gemfibrozil was found at much higher concentrations than those found by Metcalfe et al. (2003) and Hernando et al. (2004). The elimination rates found in our study were also much higher.

3.3.2.5 Enantiomer Fractions

The enantiomer fraction (EF) can be used to measure how racemic a chiral sample is (Harner et al. 2000; Meijer et al. 2001). As discussed above, the EF is calculated using the equations

$$EF = \frac{E1}{E1+E2} \tag{3.2}$$

if the enantiomer elution order is unknown, where E1 is the first eluting enantiomer and E2 is the second eluting enantiomer, or

$$EF = \frac{(+)}{(+)+(-)} \tag{3.3}$$

where (+) is the (+)-enantiomer and (-) is the (-)-enantiomer if the enantiomer elution order is known.

Any changes in EF with time are indicative of stereoselective degradation. Since many processes in the human body are stereoselective, non-racemic EFs (≠ 0.50) were expected in the WWTP influent samples. It is also believed that natural processes occurring in the wastewater treatment process will be stereoselective and further influence the EFs (Buser et al. 1999; Fono et al. 2005). To test this, standard solutions containing racemic amounts of metoprolol, atenolol, propranolol and fenoprofen were prepared in methanol. The enantiomer elution order was determined using single isomer drugs where possible. *R*-(+)-propranolol and *R*-(+)-atenolol were both determined to be the second eluting enantiomer and the EFs were calculated using equation 3.3. Enantiopure fenoprofen and metoprolol could not be obtained, and the EFs were determined using equation 3.2. The EFs for the standards were racemic as expected and are shown in Table 3.19. The EFs for the WWTP and surface water samples are presented in Table 3.16. The influents at the Edmonton WWTP on both sampling dates were racemic for all analytes. This suggests there is little or no stereoselective degradation of the pharmaceuticals in humans as they enter the WWTP. The Lac la Biche WWTP influent showed only metoprolol and atenolol. Of the two, metoprolol was non-racemic, with an EF of 0.599 ± 0.0045. This suggests metoprolol is being excreted in non-racemic proportions or that stereoselective degradation is occurring at some point prior to the influent sampling point. Whether this is occurring in the WWTP itself or is the result of biological degradation has yet to be determined. More samples should be taken from this site to confirm these preliminary results. The effluent EFs were quite different among the three WWTP samples. The first set of Edmonton WWTP samples showed racemic metoprolol but non-racemic values for atenolol, propranolol and fenoprofen. The second set of Edmonton effluent samples had racemic metoprolol, atenolol and propranolol and non-racemic fenoprofen. Finally, the Lac la Biche WWTP effluent had racemic metoprolol but non-racemic atenolol and propranolol. The differences seen among the effluents show stereoselective degradation is clearly occurring during the wastewater treatment process. However, the manner and extent to which it occurs varies between WWTP. As can be seen among the Edmonton WWTP effluents, stereoselective degradation appears to vary with time. More samples should be taken to confirm variations over time in WWTP effluent EFs.

Table 3.19 Enantiomer Fractions (EF) of 100 ng/mL Racemic Standards
N = 3
σ = standard deviation

Drugs	EF (± σ)
Metoprolol	0.519 ± 0.039
Atenolol	0.498 ± 0.029
Propranolol	0.495 ± 0.042
Fenoprofen	0.496 ± 0.031

3.4 Conclusions

The validated methods described in this thesis allow the quantification of chiral and achiral pharmaceuticals in environmental water samples down to sub ng/L concentrations. Solid phase extraction effectively cleaned up the complex matrices seen in WWTP influents and effluents and resulted in acceptable recoveries. Liquid chromatography-tandem mass spectrometry was employed for the quantification of chiral and achiral analytes in both WWTP effluents and influents and surface waters. Two chiral columns, a Chiralpak AD-RH and Chirobiotic V, gave resolution of three β-blockers and one NSAID. The method was applied to WWTP samples and surface water samples from Field Lake. All analytes were seen in the WWTP samples, although some were below the LOQ and LOD. Only four analytes were detected in the Field Lake samples. Enantiomer fractions were determined for the four chiral analytes that could be resolved. Many of the WWTP effluent samples as well as one influent sample showed definite non-racemic EFs. This thesis demonstrates that detectable concentrations of pharmaceuticals are surviving the wastewater treatment process and being discharged into the environment. We have also shown that simultaneous achiral and chiral chromatography is possible using commercially available chiral HPLC columns. This is one of the first studies to show there is stereoselective degradation occurring during the wastewater treatment process, resulting in non-racemic EFs in effluent flows. Further studies are needed to determine what environmental impact these results may have.

3.5 Future Directions

There are a number of recommendations resulting from this work that should be investigated in the future. First and foremost, the chiral chromatography problems seen for ketoprofen and fluoxetine must be addressed. The Whelk-O 1 column has proven to be somewhat unreliable. The chromatography worked well initially but degraded over time until chiral separation was not possible. After storing in methanol for a number of months, the chromatography problem seemed to subside and well-resolved peaks were seen. Again, however, the chromatography degraded and the column could not be used for the quantification of ketoprofen in the WWTP and Field Lake samples. The Pirkle-type columns, such as the Whelk-O 1, are reportedly robust with reproducible chromatography (Chen et al. 2005; Lipkowitz 2001). This was not seen in the case of this thesis. Perhaps the column needs to be replaced or perhaps the stationary phase needs to be regenerated. The problems seen with this column may also be indicative of stationary phase compression. The runs done prior to the recent decline in chromatography should be rerun in six months to see if the chiral chromatography has improved. If it has, this indicates the Whelk-O 1 is not reliable for long-term use and must be stored for long intervals of time to regenerate the stationary phase. The Chirobiotic V 2.1mm × 250 mm column recently acquired to replace the 4.6 mm × 250 mm column discussed in this thesis also needs to have its chiral chromatography investigated. The chromatography was expected to translate easily from the 4.6 mm to the 2.1 mm I.D. column. This was not the case. After many runs and much flushing of the 2.1 mm I.D. column, no appreciable chiral separation of β-blockers and fluoxetine had resulted. The peaks could be deconvoluted using the Peak Fit program, but the resolution was far from ideal and did not compare to that seen on the 4.6 mm I.D. column. The 2.1 mm I.D. must be further characterized and investigated to determine the problem with the chiral separation of the β-blockers and fluoxetine. Additionally, the effects of pH and TEAA buffer concentration on the Chirobiotic V need to be addressed. As discussed above, both the pH and TEAA concentrations have been found to strongly influence the analyte retention and stereoselective separation on this column. Using an approximate pH measured using pH paper, as was done in this work, is not satisfactory and buffer pH should be measured accurately with a pH meter each time a new buffer solution is made.

The second recommendation involves the quantification of ibuprofen. This drug was easily quantified on the Quattro LC-MS/MS during methods development. However, it was very problematic on the QTrap 2000 LC-MS/MS.

Despite many optimizations of the MS parameters, only high concentrations (> 1 µg/mL) could be seen. This is a difficult problem to fix because high concentrations (1 µg/mL) are needed during the optimization process but are inappropriate for quantifications involving environmental concentrations (ng/L). The cause of this problem is still unknown but may be related to adduct formations that have not been broken up. At high concentrations, there may be enough free ibuprofen available to consistently get the same optimization parameters. At low concentrations, adducts may have higher concentrations than the free drug and therefore not allow the free drug to be seen. The problem is not likely related to the mobile phase, since ibuprofen was not seen under any conditions on the QTrap. Changing the declustering potential (DP) is the most logical place to start because the DP helps to decluster ions and keep them apart. The capillary temperature, ionspray voltage and collision energy may also need further investigation. Alternatively, perhaps a fragment of ibuprofen should be the target ion, instead of the parent MRM transition, if adducts are truly the problem.

The third recommendation is to investigate the importance of using a surrogate standard during SPE. The current method uses only internal standards added before analysis to account for instrumental variations. The surrogate standard can be used to account for method variations. A potential candidate is deuterium labelled naproxen. It may also help account for the high recoveries seen during WWTP sample extraction. Post-column addition could also be investigated to help counter any ion suppression. There was some ion suppression seen for the internal standards in the WWTP samples. This can be problematic because the ion suppression may not be affecting the analytes to the same extent. The β-blockers are quite similar in structure so ion suppression may not be a big problem when using (+)-levobunolol as the internal standard but is likely problematic when using D_{10}-carbamazepine as the internal standard. Additionally, fluoxetine and caffeine are not as structurally similar to (+)-levobunolol or D_{10}-carbamazepine and may not be suppressed to the same extent. The retention times are also not identical, and the different matrix composition can lead to differing amounts of ion suppression.

The fourth recommendation is to find a more appropriate internal standard for caffeine, such as deuterated caffeine. This may help confirm whether the high concentration seen in the environmental samples are truly due to that much caffeine being present or if it is related to a problem with the suitability of the internal standard. At the time of this writing, D_3-caffeine had arrived and will be incorporated into future analyses.

The fifth recommendation is to investigate whether naproxen is undergoing stereoselective degradation during the wastewater treatment process. *R*-(-)-naproxen, which is not used in pharmaceutical preparations, is now available from Sigma-Aldrich and can be used to confirm the presence of this enantiomer in the WWTP samples. This would provide more chiral separation data and allow the determination of EFs for naproxen. If the single enantiomers of fenoprofen and metoprolol become available, they should be obtained and used to determine the elution order on their respective columns.

The final recommendation is to continue with the analysis of WWTP influents and effluents and surface waters to confirm and expand on the results presented in this dissertation. Samples should be taken from Lac La Biche and compared to those found in Field Lake to see if the drugs are present in similar concentrations in both. Samples should also be taken from the North Saskatchewan River at Edmonton near the outfall of the WWTP and compared to the concentrations found in the WWTP samples. The results presented in this thesis suggest there may be differences in EFs over time within the Edmonton WWTP. This line of investigation should be continued to see if the EFs truly do vary over time and to see if similar results are seen in the Lac La Biche WWTP.

3.6 References

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